PRACTICAL PLANT BIOCHEMISTRY

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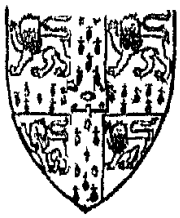

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PRACTICAL PLANT BIOCHEMISTRY

BY

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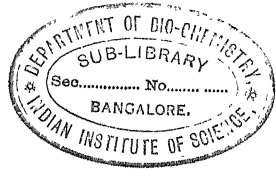

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PREFACE

THIS book is intended primarily for students of Botany Such a student's knowledge of plant products is usually obtained, on the one hand, from Organic Chemistry on the other hand, from Plant Physiology, between these two standpoints there is a gap, which, it is hoped, the following pages may help to fill It is essentially a text-book for practical work, an aspect of Plant Biochemistry which has received up to the present time very little consideration in teaching A number of experiments have been devised and have been actually tested in practical classes These experiments should enable a student to extract from the plant itself the chemical compounds of which it is constituted, and to learn something of their properties An elementary knowledge of Organic Chemistry on the part of the student has been assumed, as it appeared superfluous to incorporate the material which has already been so amply presented in innumerable text-books

My sincerest thanks are due to Dr F. F Blackman, F R.S , for criticism and many suggestions throughout the writing of the book I am further indebted to Mr H Raistrick, M.A , for help in various ways, especially in reading the proof-sheets I wish, in addition, to express my gratitude to Professor F. G. Hopkins, F.R S , for the great interest he has always shown in the subject and for his kind and stimulating advice in connexion with the scheme of teaching presented in the following pages

M. W O

CAMBRIDGE,
February, 1920.

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CHAPTER I

INTRODUCTION

This chapter should be re-read after the remaining chapters have been studied

ALL plants are made up of a complex organized mixture of chemical substances, both organic and inorganic As a preliminary to the study of plant chemistry, the student should realize that the chemical compounds which make up the living plant may be approximately grouped into the four following classes Thus, in later chapters, when reference is made to any plant product, it will be understood, broadly speaking, to which class it belongs, and what relationship it bears to other chemical compounds

The main classes may be enumerated as follows ·

(1) *Carbohydrates* These contain only carbon, hydrogen and oxygen The simplest members are the sugars, which are aldehydes and ketones of polyhydric alcohols of the methane series of hydrocarbons. The more complex carbohydrates, such as starch, cellulose, dextrins, gums and mucilages, are condensation products of the simpler sugars The sugars are found in solution in the cell-sap of living cells throughout the plant. Cellulose, in the form of cell-walls, constitutes an important part of the structure of the plant, and starch is one of the most widely distributed solid "reserve materials"

(2) *Fats* These also contain only carbon, hydrogen and oxygen Chemically they are glycerides, that is glycerol esters of fatty acids. These acids are derived from two series of hydrocarbons, i.e. the methane series and the olefine series, and they usually contain a large number of carbon atoms The fats occur as globules deposited in the cells, especially in the tissues of seeds where they form reserve materials, though they also occur in other parts of plants. The acid components of the fats are also found in the free state.

The carbohydrates and the fats both belong to the aliphatic series of organic compounds, that is to the series in which the carbon atoms are united in chains.

(3) *Aromatic compounds.* These are characterized by having the carbon atoms united in a ring, as in benzene. They may contain more than one carbon ring, and, moreover, aliphatic groupings may be attached

to the carbon ring as side-chains The number of aromatic substances is very great, and every plant contains representatives of the class Some members are widely distributed, others, as far as we know are restricted in their distribution, and may be peculiar to an order, a genus or even a species This class contains the various phenols, i e hydroxy-derivatives of benzene, such as phloroglucin, and the corresponding acids, i e hydroxy-derivatives of benzoic acid, such as gallic and protocatechuic acids Just as in the case of the carbohydrates, where simpler compounds may become more complex by condensation, so the aromatic acids, aldehydes, and their derivatives may be condensed to form more complex substances, such as tannins Other members containing more than one benzene ring are the water-soluble yellow, red, purple and blue pigments of plants, the yellow being hydroxy-flavones and flavonols, the remainder anthocyanins The simplest aromatics occur in true solution in the cell-sap throughout the plant, and the more complex ones exist in the colloidal state

Another section of aromatics is represented by the "essential oils" They are heterogeneous in chemical composition, though they are chiefly represented by the hydrocarbons of the terpene series They also include various alcohols, aldehydes and ketones They have no relationship with the true fats, but are responsible for many of the well-known scents of plants, as examples may be mentioned limonene in lemons, pinene in Conifers, borneol in Thyme and Rosemary, menthol in Peppermint and camphor in the Camphor tree (*Laurus Camphora*)

(4) *Proteins* This large class contains substances which are in many cases built up of groupings from both the aliphatic and aromatic series It includes not only the proteins but also their simpler derivatives, the albumoses, peptones and polypeptides In this case, as before, the simplest derivatives, known as the amino-acids, are synthesized by condensation to form the polypeptides, peptones, albumoses and proteins, in a series of increasing complexity. The amino-acids are compounds, either of the aliphatic or aromatic series, in which one or more hydrogen atoms are replaced by the radicle NH_2 They are soluble and crystalline, but after condensing together, the final product, the protein, only exists in either the solid or the colloidal state Proteins, in the latter condition, constitute the bulk of the complex material, protoplasm, in the solid state, in the form of grains and granules, they occur as reserve material in the cell.

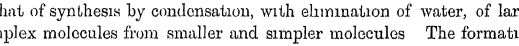
The above-mentioned classes will be dealt with in greater detail in the later chapters There are, of course, a large number of other

substances in the plant, some of which may be included with the above, while others form additional small classes, as for instance, the alkaloids others, again, bear no close relationship to any class, such as the all-important pigment, chlorophyll.

In order to appreciate the subject of plant chemistry, the plant, which is familiar as a botanical entity, must be interpreted in chemical terms. The principal classes of the more essential and widely distributed compounds found in plants have already been indicated on the broadest basis, so that they may now be referred to without additional comment.

From the botanical point of view, the plant may be regarded as a structure composed of many living protoplasmic units enclosed in cell-walls and combined together to form tissues. There are also certain tissues, known as dead tissues, which assist in giving rigidity to the plant All these structural elements can be translated into terms of chemical compounds

One of the chemical processes most frequently met with in the plant is that of synthesis by condensation, with elimination of water, of large complex molecules from smaller and simpler molecules The formation of cellulose, for instance, is a case in point. Cellulose has the composition $(C_6H_{10}O_5)_n$ and, on hydrolysis with dilute acids, it yields glucose as a final product Hence it is concluded that the complex molecule of cellulose is built up from the simpler carbohydrate by condensation. The synthesis of proteins from amino-acids affords another example These acids contain either an aliphatic or aromatic nucleus (let it be R), and one or more carboxyl and amino groups. Condensation takes place in the plant, with elimination of water, according to the following scheme.



The products of such condensation, the proteins, vary among themselves according to the number and kind of amino-acids which take part in the synthesis

Two important results arise from this process. First, the substances formed by condensation have molecules of a very large size, secondly, whereas the simple compounds, sugars and amino-acids, are soluble, crystalline and diffusible, the condensation products are either insoluble, e.g. cellulose, or exist in the colloidal state, as is the case of many proteins and other plant constituents As these very large molecules do not dialyze,

they remain where they are synthesized, and build up the solid structure of the plant, as for instance, the cell-walls

Matter in the colloidal state is of very great importance in the plant and is probably responsible for many of the properties of "living" material Thus it will not be out of place, though it will be referred to again in a later chapter, to make at this point a few remarks on the colloidal state It has been known for some time that certain metals, e g gold and silver, and also certain metallic hydroxides and sulphides, e g ferric hydroxide and arsenious sulphide, though insoluble in water under ordinary conditions, can, by special methods, be obtained as solutions which are clear to the unaided vision Such solutions are termed colloidal Investigation has shown that the matter is not present in true solution, but in a very finely divided state, i e as particles many times larger than simple molecules, but smaller than any particles obtainable by mechanical means of division. Such solutions are known as artificial colloidal solutions, but there are a number of organic substances, with *very* large molecules, such as proteins, starch, gums, agar, etc , which at once dissolve in water giving colloidal solutions The main feature of the colloidal state is that the system consists of two phases, or conditions of matter In the case of the artificial colloidal solutions first mentioned, one state is solid, the gold particles, the other state is liquid, the water The solid is known as the dispersed phase, and the water as the continuous phase, and such colloidal solutions are termed suspensoids In the case of proteins, starch, etc , both phases are liquid the dispersed phase, a concentrated solution of protein, etc ; the continuous phase, a dilute solution of protein, etc Such colloidal solutions are known as emulsoids

An important point in connexion with the colloidal state is that the molecules, or aggregates of molecules, forming the dispersed phase are so large that they exhibit some of the phenomena of surface energy, electrical charge, etc., associated with matter in mass These properties come to be of considerable importance, when we consider how large a surface is presented by matter in this state in comparison with its mass

A material in the plant upon which much interest naturally centres is the protoplasm and its nucleus It has been shown that the protoplasm consists, chemically, largely of proteins in the colloidal state. It is itself a liquid, and embedded in it are substances of various chemical constitution, in the form of granules of solid matter and also liquid globules Numerous chemical reactions are continually taking place in the

protoplasm throughout the cell, and since many of these reactions can take place both simultaneously and independently, the protoplasm must have some form of organized structure Though many phenomena of "life" may be accounted for by the physical and chemical properties of such substances as proteins, it is impossible to say, with our present knowledge, how far all "living" phenomena may yet be explained in this way

Some of the main lines of metabolic syntheses which take place in the plant will next be considered A fundamental fact which should be borne in mind is that the green plant synthesizes all the complex materials of which it is composed from the simple compounds, carbon dioxide, water and certain inorganic salts. The most important factor, perhaps, which figures in plant metabolism, is chlorophyll. The green pigments of chlorophyll are esters of complex organic acids containing the elements carbon, hydrogen, oxygen, nitrogen and magnesium They have the remarkable power of absorbing the radiant energy of the sun's rays and of transforming it into chemical energy, by means of which carbon dioxide and water are combined to form some organic compound, possibly formaldehyde, from which a simple carbohydrate is readily synthesized

If now the initial and final products of carbon assimilation be considered in detail, it will be seen that the process is one of reduction:

$$6CO_2 + 6H_2O = C_6H_{12}O_6 + 6O_2$$

This is confirmed by the fact that oxygen is evolved in the process. Moreover, the plant accumulates a store of energy, since the final product, the carbohydrate, has a higher potential energy than the system, water and carbon dioxide. Hence carbon assimilation, in addition to providing a basis of organic material as a starting-point for all the main metabolic functions, also provides a source of chemical energy by means of which reactions in other directions are brought about.

The setting free of this accumulated energy constitutes the process of respiration, which is, in reality, an oxidation of carbohydrate taking place in tissues throughout the plant. It is the converse of carbon assimilation, in that oxygen is absorbed and carbon dioxide and water are formed Thus these two processes, both so fundamental and essential to the metabolism of the green plant, are constantly taking place side by side in the same cell.

The first-formed carbohydrate, which is probably a hexose, is condensed in the plant, on the general lines we have previously indicated, to form more complex disaccharides and polysaccharides, such as maltose,

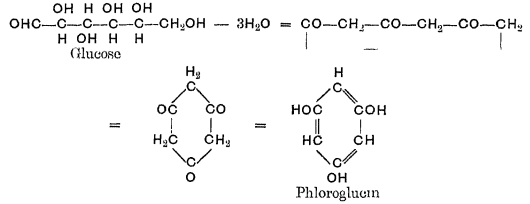
cane-sugar, starch, cellulose, etc. Some of these products, such as the disaccharides, form true solutions and may be present in the cell-sap, others, such as cellulose and starch, are present in the solid state, though they contain considerable quantities of water. Others, again, such as dextrin and gum, are present in the colloidal state. Thus, given an initial carbohydrate and a source of energy, we may proceed to indicate the other main lines of syntheses in the plant.

The next most important line of syntheses is probably that which gives rise to the nitrogen-containing constituents of the plant. Nitrogen is absorbed by the green plant in the form of nitrates and ammonium salts, but the processes which lead to the synthesis of some of the simplest nitrogen-containing compounds, such as the amino-acids, are still very obscure. Aliphatic and aromatic acids of various kinds are abundantly present in the tissues, but the reactions by which the NH_2 groups are introduced are by no means clear. There is little doubt, however, that once the amino-acids are formed, condensation takes place as already indicated, and more complex molecules, termed polypeptides, arise. Such polypeptides have now been synthesized artificially by the condensation of amino-acids. From the polypeptides, by further stages of condensation, the albumoses, peptones, and finally proteins are produced.

Another line of syntheses is that which leads to the production of the fats and allied substances. The fats are mainly glycerides of acids of the methane and olefine series, such as butyric, palmitic and oleic acids. Like all other plant products the fats must either directly or indirectly arise from the carbohydrates. There is evidence that the origin is fairly direct, as, for instance, in fatty seeds when the fats take the place of sugars in ripening. The sugars, as we know, are aldehydes of the polyhydric alcohols of the methane series. It has been suggested, though the actual stages have not been ascertained, that by various oxidation and reduction processes, the sugars yield fatty acid residues which then condense to form the fatty acids of high molecular weights present in fats. By a converse process, the fats, especially when they are stored as reserve materials in seeds, are broken up, and sugars are again formed which pass to other parts of the germinating seedling, and are there used in other synthetic processes.

A third main line of syntheses is that which gives rise to the aromatics of the plant. Since no ring compound is absorbed by the green plant, it follows that by some process the aliphatic structure must be transformed into the aromatic. Thus, for instance, the trihydric phenol,

phloroglucin, might at some stage be formed from a hexose by conversi of the aliphatic chain into a closed ring



There is evidence that aromatic compounds, such as phloroglucin tannins, flavones and anthocyanins are synthesized in the leaves, an that sugar-feeding, by floating leaves in sugar solutions, leads to th increase of aromatics in the tissues. When the ring structure has bee once synthesized, further changes can take place either by the additio of side-chains to the ring or by the condensation of two or more ring In this way the great multitude of aromatic products present in th higher plants may arise.

Thus the cell can be pictured as a colloidal solution of protein endowed with the properties of matter in mass and surrounded by a permeable cell-wall of cellulose. The colloidal solution contains liqui and solid particles of very varied chemical composition In the protoplasm are spaces, vacuoles, filled with cell-sap also containing many and various substances in solution. Throughout the protoplasm, whicl probably has an organized structure, many kinds of chemical reaction are continually in progress, some being the converse of others, as fo instance those of oxidation and reduction which can take place side by side in the same cell.

Next will be considered the chemical reactions by which the various metabolic changes in the plant are brought about How are these processes controlled and how do they take place?

There is a large group of organic substances, termed enzymes, many of which are present in every plant. They have a certain characteristic in common, i.e they bring about chemical reactions in the plant without undergoing any permanent change: in other words they are organic catalysts. Many of these reactions, which take place in the cell at ordinary temperatures with considerable rapidity need prolonged heating at high temperatures when brought about by artificial means. Enzymes

can generally be extracted from the plant by water, especially if the tissues are thoroughly disintegrated Their chemical constitution is at present unknown, and they are usually destroyed by temperatures greater than 60°C Moreover, many of the processes which they control in the plant can be brought about by them *in vitro* under suitable conditions, and it is by means of such experiments that information as to their rôle in plant metabolism has been ascertained The majority of known enzymes control both hydrolysis and its converse, synthesis by condensation with elimination of water, but under artificial conditions hydrolysis most frequently occurs The enzyme, diastase, for instance, found in all starch-containing plants hydrolyzes *in vitro* starch to dextrin and maltose Similarly the enzyme, maltase, hydrolyzes maltose into glucose Other enzymes hydrolyze proteins into amino-acids, and others, again, hydrolyze fats into fatty acids and glycerol

Until fairly recently the fact escaped notice that such reactions are reversible, and that these enzymes *in situ* in the plant may, according to the conditions, control not only the hydrolytic but also the corresponding synthetic process The latter may also be brought about, though not readily, *in vitro* This, and other evidence, leads us to believe that enzymes in the plant control the reactions in both directions

Hydrolysis, and synthesis with elimination of water are not however the only processes catalyzed by enzymes There is another type of these catalysts, the oxidizing enzymes, which bring about oxidation of substances in the plant, notably of aromatics In addition, there is the enzyme, zymase, which decomposes sugar with the production of alcohol and carbon dioxide

The question which now arises is—How many reactions in the plant are catalyzed by enzymes? It is conceivable that a greater number of enzymes may exist than are at present known, but that they are unable to be extracted by our present methods of isolation A certain number of reactions probably take place in the cell-sap between the substances in solution; others are catalyzed by enzymes which are supposed to be intimately connected with the protoplasm, but there are an enormous number to which there is at present no clue as to how they are brought about, such, for instance, as the synthesis of carbohydrates from carbon dioxide and water, and the formation of the benzene ring from the open carbon chain Such processes are usually said to be controlled by the "living protoplasm," but what exactly is the significance of this expression is at present beyond our knowledge.

Finally, also, little is known of the question as to how the various

lines of metabolic syntheses in different parts of plants are regulated and correlated with each other Some of the phenomena involved are shortly outlined as follows There is undoubtedly, under suitable conditions, a constant synthesis of sugars in the leaves. In all probability aromatic substances are also synthesized in the same organs, for there is evidence that there is an increase of these compounds in the leaf if translocation through the petiole is prevented It is possible that amino-acids also are formed in the leaf. The above products are constantly translocated to the growing organs as material for growth. They may, nevertheless, be temporarily stored in the tissues where they have been synthesized, and of this there is evidence in at least one case, e.g starch in the leaf But, apart from the immediate use for growth, there is in practically every plant, some tissue where, owing to some unknown stimulus (causing probably changes in permeability of the cell-membranes), accumulation of compounds occurs This accumulation is characteristic of organs from which growth will take place when it is impossible for the plant to obtain fresh supplies by carbon assimilation, as, for example, of bulbs, rhizomes, tubers, buds, seeds, fruits and woody tissues In these cases, in due time, the products stored supply the growing shoots.

During storage, simple sugars, amino-acids, etc. have been condensed to form insoluble, colloidal, or large molecules of starch, fats, aleurone, cane-sugar, etc These will remain until they are hydrolyzed by enzymes when they can supply the growing shoots. Such stores are termed "reserve materials" The actual stimuli involved in bringing about and regulating this storage are unknown, but they are probably connected with the life cycle of the particular plant under consideration and its adaptation to external conditions.

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CHAPTER II

THE COLLOIDAL STATE

MANY of the substances of which the plant is built up exist in the living ell in the colloidal state, and it is therefore important that some account hould be given of this condition of matter

There are many organic products found in the plant (and also in the nimal), such as starch, various proteins, gums, etc., that apparently dis- olve in water, giving a solution which, as a rule, only differs from an rdinary solution by being opalescent In addition, it has been known or a long time that various inorganic substances, such as sulphides of rsenic and antimony, hydroxide of iron, and also certain metals (gold, lver), can, by special methods, be obtained in "solution," though in rdinary circumstances they are quite insoluble. The above examples re representative of colloidal solutions

A property which all the above solutions possess is that the substance issolved will not pass through a parchment membrane, i e will not ialyze, whereas if a solution of sodium chloride in water is separated om pure water by a parchment membrane, the salt will pass through ie membrane until the concentration of the sodium chloride is equal i either side of it

The conclusion drawn from investigations of various kinds is that in ie colloidal solutions the substances dissolved exist in the state, either aggregates of molecules, or of very large molecules, and hence are iable to pass through the pores of the parchment

Moreover, certain distinctions can be drawn between colloidal solu- ons some, like those of gold, silver, metallic sulphides, hydroxides and fact most inorganic substances, are very sensitive to the presence of iall amounts of inorganic salts, i e electrolytes, and are precipitated them, but will not as a rule go into solution again Also such col- dal solutions are very little more viscous than pure water. The organic bstances, on the other hand, are only precipitated from colloidal solu- ons by comparatively large quantities of electrolytes The viscosity, oreover, of these solutions is greater than that of water, and is, in fact, nsiderable, even if the percentage of dissolved matter is small.

Hence two terms have been employed for the above-mentioned types of colloidal solutions those of gold, silver, etc, are termed *suspensoids* (suspensoid sols) those of starch, proteins, etc, *emulsoids* (emulsoid sols)

The essential feature of both forms is that they are systems consisting of two phases, or conditions of matter, known respectively as the "dispersed" phase and the "continuous" phase

A *suspensoid* may be defined as having a *dispersed phase* composed of ultramicroscopic particles or aggregates of molecules suspended in a *continuous phase* composed of a liquid

An *emulsoid* may be defined as having a *dispersed phase* composed of ultramicroscopic drops of a highly concentrated solution of the substance suspended in a *continuous phase* composed of a dilute solution of the same substance

As a rule, therefore, the difference between a suspensoid and an emulsoid is that, whereas in the former the liquid is restricted to the continuous phase, and the solid to the dispersed phase, in an emulsoid both phases are liquid, though containing different proportions of the dissolved substance

The terms suspensoid and emulsoid are used on account of the resemblance of these states of matter respectively to suspensions and emulsions If microscopic particles of a solid are shaken up in water, what is known as a suspension is obtained, in time, however, the solid particles, if heavy enough, will settle and separate from the water, and the whole process can be repeated. Thus a suspension differs from a suspensoid solution in that the latter is stable, though, if precipitated, the reaction is usually not reversible.

If two liquids which are insoluble in each other, such as oil and water, are shaken up together, finely divided drops of oil in water are obtained. This is known as an emulsion. In time, however, the oil separates from the water, because the tension on the films of water separating the oil drops, when in contact, is too great, and they break, with the result that the oil drops coalesce But if, instead of water, a solution of soap, saponins, or certain other substances is used, the surface tension of the water is so lowered that the films of soap solution separating the oil drops are permanent, and a system is obtained consisting of minute drops of oil separated by soap solution This system resembles an organic colloidal solution, as, for instance, that of protein in which we suppose a concentrated solution of protein exists in drops separated by a dilute solution of protein. Milk and latex constitute natural emulsions

Expt 1 *Formation of a suspension* Precipitate a solution of barium chloride with some sulphuric acid and shake up well the fine precipitate of barium sulphate Note the gradual settling of the precipitate

Expt 2 *Formation of an emulsion* Take a drop of olive oil in a test-tube and half fill the tube with alcohol Shake well and pour into a beaker of water A fine white emulsion of oil in water will be formed from which the oil will not separate By this method the oil is obtained in such small drops that stability is ensured

Take about equal quantities of olive oil in two test-tubes and add an equal quantity of water to each To one tube add a drop or two of 10 % caustic alkali solution Shake both test-tubes well An emulsion is formed in both, but in the tube without alkali the oil will separate out on standing In the other tube the emulsion is permanent This is due to the fact that the olive oil (unless specially purified) contains some free fatty acid The latter forms soap with the alkali (see p 82) and renders the emulsion permanent

Expt 3 *Preparation of suspensoid sols* (*a*) *Gold* Take two 100 c.c measuring cylinders and thoroughly clean them with nitric acid, and afterwards wash well with freshly distilled water In one make a 0 5 % solution of tannic acid (using the purest sample obtainable) in water In the other, 2 c c of commercial 1 % gold chloride are made up to 100 c c with water Use freshly distilled water in both cases Mix equal portions of the two solutions in a clean beaker A purple colloidal solution of gold will be formed If three parts of the chloride solution are mixed with one part of the tannin solution, and both solutions heated before mixing, a red colloidal solution is obtained (*b*) *Silver* Take 5 c c of a 1 % solution of silver nitrate and add dilute ammonia solution until the precipitate first formed just disappears, and then dilute with 100 c c of water Mix equal volumes of this solution and the tannic acid prepared for (*a*) A colloidal solution of silver will be formed which is clear brown by transmitted light, but has a green fluorescence by reflected light. (*c*) *Ferric hydroxide* Take 5 c c of a filtered 33 % solution of ferric chloride and pour into 500 c c of boiling distilled water in a beaker A colloidal ferric hydroxide sol is formed and the colour changes to a deep brown-red The yellow solution of ferric chloride is decomposed by excess of water with the production of a soluble colloidal form of ferric hydroxide, and hydrochloric acid is set free. (*d*) *Arsenic trisulphide.* Take 2 gms. of arsenious acid and boil with 150 c c of distilled water, filter and cool. Then pass sulphuretted hydrogen through the solution A colloidal solution of the sulphide is formed which is orange, with a greenish surface

The above sols should be kept for further experiment [see Expt 8]

Expt 4 *Preparation of emulsoid sols* (*a*) *Starch* Weigh out 2 gms. of dry starch, and mix well with a little cold distilled water Boil 100 c c. of distilled water in a flask, and, when boiling, pour in the starch paste and boil for a few minutes longer, stirring well all the time A colloidal solution of starch is obtained which is faintly opalescent It is not affected by heating and does not change its state on cooling. (*b*) *Gum arabic* Make a 5 % solution of gum arabic by boiling 5 gms. with 100 c.c. of distilled water. Note that a sticky or viscous solution is formed which froths on shaking (*c*) *Protein* Weigh out 10 gms of white flour and add 100 c.c. of distilled water Let the mixture stand for 2 or 3 hours and then filter. The extract contains protein Note that the solution froths on shaking. (*d*) *Soap* Make a 5–10 % solution

of soap in distilled water It is opalescent and froths strongly. (*c*) *Chlorophyll* [See Expt 32]

The above sols should be kept for further experiment [see Expt 9]

Expt 5 Dialysis of starch and salt solution Make a 2 % solution of starch in water, as in Expt 2, and mix it with an equal volume of a 2 % solution of sodium chloride in water Pour the mixture into a parchment dialyzer and dialyze in a beaker of distilled water (The dialyzer should first be carefully tested to ascertain if there be a leak) Test the liquid in the beaker with solutions of both iodine and silver nitrate Some precipitate of chloride will be given, but no blue colour with iodine After 24 hours, test the liquid again There will be an increased amount of silver chloride formed, but a negative result with iodine On addition of iodine to the liquid in the dialyzer, a blue colour is obtained Hence we may assume that the colloidal starch does not pass through the membrane

Some substances, such as gelatine (animal) and agar (vegetable), are only in the emulsoid condition at a raised temperature When cold they set to form a "gel," in which the particles of the dispersed phase are no longer separate but united to make a solid. Silicic acid, the best known inorganic emulsoid, also sets to a gel on standing, either spontaneously or on addition of electrolytes. It is of classical interest since it was the substance largely used by Graham, the first worker on colloids.

Expt 6 Preparation of gels (*a*) *Agar* Weigh out 2 gms of agar and put it to soak in 100 c c of distilled water for an hour or two Then boil . the agar gives a thick opalescent solution (sol) which sets to a gel on cooling On warming, the gel again becomes a sol, and, on cooling, again sets to a gel Thus the change is a reversible one Agar is a mucilage which is obtained from certain genera of the Rhodophyceae (see p 49) (*b*) *Silicic acid* Weigh out 20 gms of commercial "water-glass" syrup (a concentrated solution of sodium silicate) and dilute with 100 c.c. of freshly boiled distilled water (free from carbon dioxide). Pour 75 c c. of this solution into a mixture of 25 c c of concentrated hydrochloric acid and 75 c c. of water Dialyze the mixture in a parchment dialyzer against running water for 3–4 hours If to the dialyzed liquid a little very dilute ammonia is added, a gel will be formed in the course of a few hours. In this case, however, the process is irreversible, that is the gel cannot be reconverted again into the sol.

An interesting point in connexion with the colloidal state is that emphasized by Ostwald, i c. that this condition is a *state*, not a *type*, of matter Further, substances in the colloidal state do not constitute a definite class. It is reasonable to suppose that all substances which exist in the colloidal state can, under suitable conditions, also exist in the crystalline state, and *vice versa*. Further, the continuous phase is not always water. Sodium chloride, which is a very definite crystalloid, can be obtained in the colloidal state in petroleum ether. Most metals, even the alkali metals, have been obtained in colloidal solution also a great many metallic oxides, hydroxides and sulphides.

The colloidal phases so far dealt with can be tabulated as follows[1]:

disperse	*continuous*	
liquid	solid	gels
solid	liquid	suspensoids
liquid	liquid	emulsoids

Some of the properties of colloidal solutions may now be considered A point that has already been emphasized in the previous chapter is that the surface of particles in the colloidal state is very great in proportion to their mass Such particles, moreover, unlike ions and small molecules in true solution, possess the properties of the surfaces of matter in mass, as, for instance, those connected with surface tension, electrical charge, etc, and these are especially marked on account of the proportionately large surfaces involved Other properties are their inability, as a rule, to exert an osmotic pressure, to raise the boiling point, and to lower the freezing point of water. Some of the metallic suspensoids are characterized by their colour, this being red, purple or blue as in the case of gold sols

An apparatus, by means of which the colloidal state can be demonstrated ocularly, is the ultramicroscope This is a special form of microscope in which a powerful beam of light is directed upon a colloidal solution, which is then seen to contain a number of particles in rapid motion. When analyzed by special methods, this motion has been found to be identical with that shown by much larger, though still microscopic, particles, which has been termed Brownian movement

Expt 7. *Demonstration of Brownian movement of microscopic particles* Mount a little gamboge in water and examine under the high power of a microscope The particles will be seen to be in rapid motion.

It has been shown that Brownian movement is the outcome of the movement of the molecules of the liquid in which the particles are suspended This movement is one of the factors which keeps the sol stable and prevents the particles from "settling" as in the case of a true suspension.

Another factor tending to keep the sol stable is the electrical charge borne by the particles. It is commonly known that there is usually a difference of potential between the contact surfaces of phases If the

[1] There are also the following combinations (Bayliss, 1)

disperse	*continuous*	
gas	liquid	foam
liquid	gas	fog
solid	gas	tobacco smoke
solid	solid	ruby glass (colloidal sol of gold in glass)

particles in a colloidal solution all have the same charge, then they will tend to repulse one another mutually It is found that the particles are charged, but the origin of the charge is not always clear. Sometimes if the substance in colloidal state is capable of electrolytic dissociation, the charge may arise in this way Substances, however, as already mentioned, which are not dissociated may also bear a charge, and most frequently it is a negative one It follows, then, that when an electrolyte is added to a colloidal solution, the charges on the colloidal particles are neutralized by the oppositely charged ions of the electrolyte, and they coalesce together and are precipitated

As regards their behaviour to electrolytes the two classes, suspensoids and emulsoids, are very different The suspensoids are very sensitive to traces of electrolytes, and, as they usually have a negative charge, it is the cation of the electrolyte which is the active ion, and of such, less of a divalent ion, than of a monovalent ion, is needed for precipitation and still less of a trivalent ion

The emulsoids are far less sensitive to electrolytes in solution than the suspensoids, in fact, electrolytes, such as neutral alkali salts, must be added in very large quantities to emulsoids before precipitation takes place Also, as a rule, whereas the precipitation of suspensoids is irreversible, that of emulsoids is reversible, that is, they pass into solution again on addition of water In the case of an emulsoid in neutral solution this form of precipitation, unlike that of the suspensoids, may be regarded as consisting of two processes. First, a process analogous to that of "salting out" of soaps, esters, etc., in organic chemistry, which is, in effect, a withdrawal of water from one phase into another Secondly, the precipitation is also affected to some extent by the valency of the ions of the salt used in precipitation.

When, however, a neutral solution of such an emulsoid as protein is made either slightly acid or alkaline, its behaviour towards neutral salts becomes altered. The precipitating power of salts in acid or alkaline medium is now in accordance with that on suspensoids. In alkaline solution the coagulating power of a salt depends on the valency of the cation, in an acid solution it depends on the valency of the anion.

The behaviour of proteins in acid and alkaline media is undoubtedly due to the fact that they are built up of amino-acids containing both amino and carboxyl groups Such molecules may behave either as an acid or a base with the formation of salts These are subject to electrolytic dissociation and hence acquire an electric charge Such substances have been termed "amphoteric electrolytes" (see p. 120)

Expt 8 Precipitation of suspensoid sols by electrolytes The sols of gold, silver and arsenious sulphide carry an electro-negative charge hence they are most readily precipitated by di- or tri-valent positive ions, such as Ba″ or Al‴ Add a few drops of barium chloride solution to the three sols (Expt 3) respectively, and note that they are precipitated, though some time may elapse before the precipitation is complete The ferric hydroxide sol, on the contrary, carries a positive charge Hence it is most readily precipitated by a sulphate or phosphate If a drop of sodium sulphate solution is added while the sol is hot, it is immediately precipitated

Expt. 9 Precipitation of emulsoid sols by electrolytes Saturate the starch, protein and soap solutions prepared in Expt 4 with solid ammonium sulphate Precipitation takes place in each case, and it is seen how large a quantity of electrolyte is needed for the "salting out" of emulsoid sols Filter off the protein precipitate and suspend in distilled water It will go into solution again, showing that the reaction is reversible

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CHAPTER III

ENZYME ACTION

SOME indication has been given in the previous chapter of the large number of complex processes which take place in the plant, and it has been mentioned that many of these are controlled by enzymes

The most remarkable feature in connexion with the chemical processes of plant metabolism is the ease and rapidity with which, at ordinary temperatures, chemical reactions take place, when under artificial conditions they need a much longer time and higher temperatures

It has been found that many of the chemical reactions in the plant can be brought about *in vitro* on addition of certain substances which can be extracted from the plant These substances are known as enzymes. It is the property of enzymes that they are able to accelerate reactions which, in their absence, would only take place very slowly The enzyme cannot initiate these reactions and does not form part of their final products

Some inorganic substances have the same property of accelerating reactions, and such substances are termed catalysts For example, when water is added to ethyl acetate, the latter begins to be decomposed slowly into ethyl alcohol and acetic acid:

$$\text{ethyl acetate} + \text{water} \longrightarrow \text{ethyl alcohol} + \text{acetic acid},$$

but if, in addition, some hydrochloric acid is added, hydrolysis takes place with much greater rapidity, and at the end of the reaction the hydrochloric acid is found unchanged Hence in this case hydrochloric acid is an inorganic catalyst. Many other similar instances are known as, for example, the catalyzing effect of a small quantity of manganese dioxide which brings about the liberation of oxygen from potassium chlorate at a much lower temperature than by heat alone.

By analogy, therefore, an enzyme may be defined as an organic catalyst produced by the plant.

Another point in connexion with the above-mentioned reaction of water with ethyl acetate, is the fact of its being representative of the type known as reversible. After a certain amount of acetic acid and ethyl alcohol has been formed, these recombine to form ethyl acetate until in time a certain point of equilibrium is reached. Since the same

point of equilibrium is reached whether hydrochloric acid is used or not, it is obvious that the hydrochloric acid accelerates the reaction in both directions

$$\text{ethyl acetate} + \text{water} \rightleftarrows \text{ethyl alcohol} + \text{acetic acid}$$

Such a reaction is termed a reversible one Many of the processes accelerated by enzymes in the plant are reversible, and there is reason to believe that the enzyme accelerates the reaction in both directions

The substance upon which the enzyme acts is termed the substrate, and it is supposed that some kind of loose combination occurs between these two substances The enzyme is unaltered when the reaction is complete, unless it is affected by the products formed

The enzymes are very widely distributed and form constituents of all living cells, though all tissues do not necessarily contain the same enzymes

There is no doubt that many enzymes are specific, in which case an enzyme can only accelerate one reaction, or one class of reaction. We cannot be sure that any enzyme is specific and different from all others, until it has been proved that it accelerates one process which is incapable of being accelerated by any other enzyme It is possible that some enzymes, to which separate names have been given, are really identical

Most of the plant enzymes are soluble in water and dilute glycerol and sometimes in dilute alcohol Some can be extracted by simply macerating the tissues with water, others are more intimately connected with the protoplasm, and are only extracted if the protoplasm is killed by certain reagents, of which those most frequently employed are toluol and chloroform These substances kill the protoplasm and do not, in many cases, affect the enzyme After the death of the protoplasm, the enzymes are more readily extracted from the cell From aqueous solutions enzymes can usually be precipitated by adding strong alcohol.

The majority of enzymes are destroyed by raising the temperature above 60° C *In vitro* then reactions are generally carried out most rapidly between the temperatures of 35–45° C.

In performing experiments with enzymes *in vitro*, it is always necessary to add an antiseptic, otherwise the reaction to be studied will be masked or entirely superseded by the action of bacteria unavoidably present Toluol and chloroform mentioned above, as well as thymol, may be used These reagents prevent any bacterial action from taking place Some enzymes, however, are susceptible to chloroform, as, for instance, maltase

The chemical nature of enzymes is at present unknown, because it is difficult to purify them without destroying them, and hence to obtain them of sufficient purity for chemical analysis They were originally thought to be proteins, but with the improvements in methods for purification, it has been found that the protein reactions disappear, although the enzyme activity does not decrease In solution they exist in the colloidal condition

The questions as to their origin and their relation to the protoplasm cannot yet be answered with any certainty It is also impossible to say whether the majority of chemical processes in the plant are catalyzed by enzymes.

A feature of enzyme action which is of considerable interest and which has already been mentioned is the question as to whether enzymes catalyze the synthetic as well as the hydrolytic reaction There is evidence that this is so, since, in many cases, the hydrolysis is not complete If the enzyme were a catalyst in one direction only, the reaction would be complete Further evidence is supplied by the fact that, under suitable conditions, i.e. strong concentration of the substances from which synthesis is to take place, certain syntheses have been carried out *in vitro* As an example may be quoted the synthesis of maltose from a concentrated solution of glucose by maltase (Bayliss, 2)

In the living cell it is supposed that the hydrolysis and synthesis are balanced On the "death" of the protoplasm, which may be caused by mechanical injury, vapour of chloroform or toluol, etc (Armstrong, 7, 8), the reactions catalyzed by enzymes cease to be balanced and proceed almost always in the direction of hydrolysis and the splitting up of more complex into simpler substances. This phenomenon is obvious when any of the products can be recognized by smell or colour, as, for instance, the smell of benzaldehyde on injuring leaves of plants containing cyanogenetic glucosides (see p 146), or the production of coloured oxidation products when some of the aromatic glucosides are decomposed (see p 113)

If plant tissues are disintegrated, and the mass is kept at a temperature of about 38°C., the above-mentioned hydrolytic processes continue to be catalyzed by the enzymes present until equilibrium is reached, which will be near complete hydrolysis, especially if water has been added Such a process is termed "autolysis."

The chief plant enzymes may be classified under the following headings:

A. *Hydrolytic enzymes.* Those constitute by far the greater number of known enzymes. In their activity they either add or eliminate water.

According to the substances upon which they act (the substrates) they may be further sub-classified as follows

(1) Fat-splitting enzymes, which hydrolyze fats into fatty acids and glycerol lipase

(2) Carbohydrate-splitting enzymes

Diastase, which hydrolyzes starch into dextrin and maltose

Invertase, which hydrolyzes cane-sugar into dextrose and laevulose

Maltase, which hydrolyzes maltose into dextrose

Inulase, which hydrolyzes inulin into laevulose

Cytase, which hydrolyzes hemicellulose into mannose and galactose.

Pectinase, which hydrolyzes pectic compounds into arabinose

(3) Glucoside-splitting enzymes.

Emulsin, which hydrolyzes amygdalin into benzaldehyde, prussic acid and glucose

Myrosin, which hydrolyzes sinigrin into allylisothiocyanate, potassium hydrogen sulphate and glucose

(4) Protein-splitting enzymes (proteases)

Pepsin-like enzymes, which hydrolyze proteins into albumoses and peptones

Erepsin-like enzymes, which hydrolyze peptones into polypeptides and amino-acids.

B *Oxidizing enzymes*.

Peroxidases, which decompose peroxides and set free oxygen in the active state, probably as atomic oxygen

Catalases, which decompose hydrogen peroxide and set free oxygen in the molecular condition.

C *Fermenting enzymes* Zymase of yeast and also of higher plants, which brings about the decomposition of certain hexoses, such as glucose, with the formation of alcohol and carbon dioxide Probably a series of reactions including oxidation, reduction and hydrolysis are involved.

D *Coagulating enzymes* Pectase, which coagulates soluble pectic compounds

E *Reducing enzymes* Reductase The precise function of these enzymes is unknown.

Most of these various classes of enzymes will be dealt with in detail in connexion with the chemical substances on which they react.

An excellent demonstration of the fact that a single cell may contain all the various enzymes connected with the processes of metabolism is

afforded by the unicellular Fungus, Yeast (*Saccharomyces*), of which many species and varieties are known The feature of special interest in connexion with the Yeast plant is its power of fermenting hexoses, with the formation of alcohol and carbon dioxide, the process being carried out by means of an enzyme, zymase The reaction is generally represented as follows, though there is little doubt that several stages are involved

$$C_6H_{12}O_6 = 2CO_2 + 2C_2H_5OH$$

In addition to zymase, yeast contains the enzymes, invertase, protease peroxidase, catalase, reductase, glycogenase, carboxylase, a glucoside splitting enzyme, and some form of diastatic enzyme The carboxylase decomposes a large number of keto-acids, of which the most important is pyruvic acid $CH_3 CO COOH$ The reaction involves the formation of the corresponding aldehyde with the evolution of carbon dioxide Yeast also stores, as a reserve material, the polysaccharide glycogen, which occurs in animal tissues though it is rarely found in plants this is hydrolyzed by glycogenase into a monosaccharide Finally yeast contains invertase, and most species, in addition, maltase, but from a few species the latter enzyme is absent Hence yeasts are able to ferment the disaccharides, cane-sugar and maltose, since they can first hydrolyze them to monosaccharides

As in the case of the enzymes of other tissues, those of yeast can be made to carry out their functions after the death of the living protoplasm The method of demonstrating this is to "kill" the cells by means of drying at 25–30° C, by treatment with a mixture of alcohol and ether, or by treatment with acetone and ether. In this way the protoplasm is destroyed, but the enzymes remain uninjured Yeast treated thus has been termed "zymin"

From zymin some of the enzymes, e.g. invertase and the glucoside-splitting enzyme, can be extracted with water: other enzymes, e.g zymase and maltase, are not so readily extracted. From the living cells the enzymes are only obtained with difficulty, the extraction of yeast juice, containing zymase and other enzymes, needing, by Buchner's method, a pressure as great as 500 atmospheres.

In connexion with alcoholic fermentation by zymase, the following point is of special interest. For carrying out this process, two other substances are necessary in addition to the enzyme, i.e. a co-enzyme of unknown nature and a phosphate The separation of zymase from the co-enzyme has been accomplished by filtering expressed (Buchner) yeast juice through a special form of gelatine filter under a pressure of 50

atmospheres The phosphate and co-enzyme can also be removed from zymin by washing with water The washed residue is then found to be incapable of fermentation, as also are the washings If, however, the boiled washings are added to the washed residue, the system is synthesized and can now carry out fermentation again The chemical nature of the co-enzyme, which is thermostable, and the precise part played by it in the process, are as yet unknown (Harden, 4)

Expt 10 *Preparation of zymin* (*a*) *By alcohol and ether* For the following experiments fresh yeast should be used which has been washed several times with distilled water and dried on a filter-pump Weigh out 25 gms of yeast and stir it up well in a mixture of 300 c c of absolute alcohol and 100 c c of ether for 4–5 minutes, and then filter on a filter-pump Next wash the yeast with 50 c c of ether. Then spread it out on a piece of filter-paper and allow it to dry for one hour (*b*) *By acetone and ether* Take 50 gms of yeast and stir it into 300 c c of acetone Continue stirring for 10 minutes, and filter on a filter-pump The mass is then mixed with 100 c c of acetone for 2 minutes and again filtered The residue is roughly powdered, well-kneaded with 25 c c of ether for 3 minutes, filtered, drained and spread on filter-paper for an hour in the air It can be finally dried at 45° C. for 24 hours

Expt 11 *Action of zymase.* Fill a small test-tube with 40 % glucose solution Add 2 gms of the zymin (from Expt. 10) for each 10 c c of solution and a little toluol, and shake. Then fit a slightly larger test-tube over the mouth of the first tube Now invert A small quantity of air will rise to the top of the first tube which, however, will remain filled with the sugar solution Place the tubes either in an incubator or water-bath at 22–25° C A control set of tubes should also be arranged containing sugar solution and zymin which has been previously well boiled in a little water Bubbles of carbon dioxide will be evolved from the unboiled zymin

Expt 12 *Action of maltase* (Harden and Zilva, 12) Into two test-tubes, (*a*) and (*b*), put the following

(*a*) 20 c c of a 2 % solution of maltose + 0 5 gm of zymin

(*b*) 20 c c of the same solution of maltose + 0 5 gm of zymin which has been well boiled in distilled water.

Plug both tubes with cotton-wool, after adding a few drops of toluol, and place in an incubator at 38° C for 12–24 hrs Test the liquid in both tubes by making the osazone (see p 49) Glucosazone will crystallize out in tube (*a*), maltosazone in tube (*b*)

Expt 13 *Action of peroxidase* (Harden and Zilva, 12) Into four small evaporating dishes, (*a*), (*b*), (*c*) and (*d*), put the following.

(*a*) A suspension of 0 5 gm of fresh yeast in 10 c c distilled water + 1 c c of benzidine solution (1 % in 50 % alcohol) + 2–3 drops of hydrogen peroxide (20 vols).

(*b*) A suspension of 0 5 gm of zymin in 10 c c of distilled water + 1 c c of benzidine solution + 2–3 drops of hydrogen peroxide

(*c*) A suspension of 0 5 gm of washed zymin in 10 c c of distilled water + 1 c c

of benzidine solution + 2–3 drops of hydrogen peroxide (The zymin is washed by putting it on a double folded filter-paper in a funnel and adding distilled water from time to time 50 c c of water should be used for 0 5 gm of zymin)

(*d*) A suspension of 0 5 gm of washed zymin in 10 c c of washings + 1 c c of benzidine solution + 2–3 drops of hydrogen peroxide

A blue colour will develop in (*a*) showing that fresh yeast contains a peroxidase (see p 109) A blue colour will also develop in (*c*) but not in (*b*) and (*d*) This is explained by assuming that the zymin contains an inhibitor, not present in fresh yeast, but which is developed during the preparation of the zymin, and that this inhibitor can be washed away by water On adding the washings to the washed zymin the reaction is inhibited again

Expt 14 *Action of catalase* (Harden and Zilva, 12) Completely fill a test-tube with hydrogen peroxide (20 vols) solution which has been diluted with an equal volume of water and add 0 5–1 gm of zymin Place the thumb firmly over the mouth of the tube, invert and place the mouth under water in a small basin, clamping the tube in position A rapid evolution of oxygen takes place When the tube is about three-fourths full of gas, close the mouth with the thumb while still under water and remove the tube Plunge a glowing splint into the gas and it will re-kindle to a flame

Expt. 15 *Action of protease* Weigh out 10 gms. of white flour, and allow it to extract with 100 c c. of distilled water for one hour, shaking from time to time. Then filter on a filter-pump The extract will contain the albumin, leucosin (see p 124) Into small flasks (*a*) and (*b*) put the following.

(*a*) 40 c c of the flour extract + 1 gm of zymin + 1 c c of toluol

(*b*) 40 c c of flour extract + 1 gm of boiled zymin + 1 c c. of toluol

Shake both tubes, plug with cotton-wool and place them in an incubator at 38° C. for 48 hrs. After incubation, boil the liquid in both tubes, in order to coagulate unaltered protein, and filter To the filtrates of the respective tubes add bromine water drop by drop (see p. 138) A pink, or purplish-pink colour, due to the presence of tryptophane, will be formed in tube (*a*) Hence hydrolysis of protein has taken place Tube (*b*) will show no colour or only that due to bromine Add a *little* amyl alcohol to both tubes and shake. The alcohol will be coloured pink or purplish in the tube giving the tryptophane reaction.

Expt 16 *Action of reductase.* (Harden and Norris, 11) Take two small flasks, (*a*) and (*b*), provided with well-fitting corks and put in the following:

(*a*) 1 gm. of zymin + 20 c c. of distilled water + 0·5 c.c of methylene blue solution (made by diluting 5 c.c of a saturated alcoholic solution to 200 c.c with distilled water)

(*b*) 1 gm. of boiled zymin + 20 c c of distilled water + 0 5 c c of methylene blue solution

Cork both tubes after adding a few drops of toluol and place in an incubator at 38° C. for 3 hours The blue colour will practically disappear from tube (*a*) but will remain in tube (*b*)

The methylene blue is reduced to a colourless leuco-compound which will become blue again on re-oxidation

Expt 17 *Enzyme actions of an aqueous extract of zymin* Weigh out 2 gms of zymin and place them on a double folded filter-paper in a funnel and wash with 80 c c of distilled water With the filtrate make the following experiments

(A) *Action of invertase.* (Harden and Zilva, 12) Into two small flasks (*a*) and (*b*) put the following

(*a*) 10 c c of a 2 % solution of pure cane-sugar + 10 c c of the filtrate from zymin

(*b*) 10 c c of the same solution of cane-sugar + 10 c c of the boiled filtrate from zymin

Put both flasks in an incubator at 38° C. After 30 minutes add equal quantities (about 1–2 c c) of Fehling's solution to both test-tubes and boil (see p. 52) Tube (*a*) will show considerable reduction of the Fehling Tube (*b*) will show comparatively little reduction, that which does take place probably being due to the sugar formed by the action of glycogenase on stored glycogen

(B) *Action of the glucoside-splitting enzyme* (Caldwell and Courtauld, 9, Henry and Auld, 13) This enzyme will act upon the glucoside, amygdalin, which is present in bitter almonds, with the production of glucose, benzaldehyde and prussic acid (see p 145) Into two small flasks (*a*) and (*b*) put the following

(*a*) 20 c c of a 2 % solution of amygdalin + 20 c c of the filtrate from zymin

(*b*) 20 c.c of the same solution of amygdalin + 20 c c of the boiled filtrate from zymin

Add a few drops of toluol to both flasks and then cork, inserting, with the cork, a strip of paper which has been dipped in solutions of picric acid and sodium carbonate (see p 146) Put both flasks in an incubator at 38° C for 12–24 hours The picrate paper in flask (*a*) will have reddened Add a little Fehling's solution to the liquid in the same flask and boil. The Fehling will be reduced The liquid in flask (*b*) will only reduce Fehling slightly [see Expt A (*b*)] and the picrate paper will not be reddened.

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CHAPTER IV

CARBON ASSIMILATION

THE fact has already been emphasized that the plant synthesizes all the complex organic substances of which it is built from the simple compounds, carbon dioxide, water and inorganic salts. The initial metabolic process and the one from which all others have their starting-point is that of a synthesis of a carbohydrate from carbon dioxide and water. This synthesis can only be carried out in the light, and only in a green plant, i.e. a plant containing chlorophyll. Chlorophyll may almost be considered the chemical substance of primary importance in the organic world, for upon it depends the life of all plants and animals. Animals depend for their existence on certain complex amino-acids, some of which they are unable to synthesize for themselves, and which they derive from plants. Plants in turn are unable to exist except by virtue of the properties of chlorophyll.

The property of chlorophyll which is so important is the power it possesses of absorbing the radiant energy of the sun's rays and converting it into chemical energy by means of which a carbohydrate is synthesized. This summarizes the whole process, which, however, can scarcely be very simple, and probably consists of several reactions at present undifferentiated. If the formula for carbonic acid is compared with that of a simple carbohydrate such as a tetrose, pentose or hexose, the following relationship is seen

$$H_2CO_3 \rightarrow (H_2CO)_x \text{ where } x=4,\ 5 \text{ or } 6,$$

that is, in the synthesis of a carbohydrate a reducing reaction must take place.

Many hypotheses have been formulated as to the nature of these reactions. The one which has most frequently been advanced suggests that formaldehyde is the first product of the synthesis from carbon dioxide and water which takes place in the green plant, that the reaction involves reduction with elimination of oxygen

$$H_2CO_3 = H_2CO + O_2,$$

and that this product is later condensed to form a hexose,

$$6H_2CO = C_6H_{12}O_6$$

As the concentration of sugar increases in the cell, further condensation may take place to form starch

$$x(C_6H_{12}O_6) = (C_6H_{10}O_5)_x + x\,H_2O$$

The facts in agreement with these views are first, in most plants a volume of oxygen is given off approximately equivalent to the volume of carbon dioxide absorbed, secondly, in some plants starch, in others sugar, is known to be produced during photosynthesis The detection of formaldehyde, either in the plant or in certain systems containing chlorophyll, as a proof of its formation during photosynthesis, has been shown to be invalid (see p 36) (Jorgensen and Kidd, 2).

The value and significance of this reducing reaction is seen when it is realized that, by oxidation of the carbohydrates synthesized, energy is produced to supply the needs of the whole metabolism of the plant (see p 5)

In the chemical treatment of the subject of carbon assimilation, some of the chemical properties of chlorophyll will first be considered, and, later, its behaviour under certain conditions the chemistry, however, of the phenomenon itself is as yet unknown

The following account, as far as it concerns chlorophyll, and the accompanying experiments are taken from a *résumé* (Jorgensen and Stiles, 3) of the original work (Willstatter und Stoll, 1) upon which the entire knowledge of the subject is based

Chlorophyll.

Our knowledge of the chemistry of chlorophyll has, within recent years, been set upon a firm experimental basis (Willstatter und Stoll, 1) The results which have been arrived at may broadly be summarized as follows

In all plants examined the chloroplastids contain four pigments, of which two (termed respectively chlorophylls *a* and *b*) are green, and two are yellow. They occur in about the following proportions in fresh leaves

Green	Chlorophyll *a* ... $C_{55}H_{72}O_5N_4Mg$ ...	2	pts per 1000
	Chlorophyll *b* ... $C_{55}H_{70}O_6N_4Mg$.	$\frac{3}{4}$	„ „
Yellow	Carotin ..... $C_{40}H_{56}$	$\frac{1}{6}$	„ „
	Xanthophyll ... $C_{40}H_{56}O_2$	$\frac{1}{3}$	„ „

A point of great interest in connexion with chlorophyll is that it contains magnesium to the extent of 2·7 % but no other metal is present. **Chlorophyll a**, when isolated, is a blue-black solid giving a green-blue

solution in the solvents in which it is soluble, i.e. ethyl alcohol, acetone, chloroform, ether, carbon bisulphide, pyridine and benzene. **Chlorophyll b**, when isolated, is a green-black solid giving a pure green solution it has much the same solubilities as chlorophyll *a*. The two chlorophylls, however, can be separated by their different solubilities in methyl alcohol Both can be obtained in microscopic crystals

Carotin crystallizes in orange-red crystals, and xanthophyll in yellow crystals

In the chloroplastids these pigments occur mixed with various colourless substances, fats, waxes, and salts of fatty acids

When chlorophyll is spoken of, it will be understood to refer to the green pigments and not to the yellow.

The *pure* pigments, when *isolated*, are readily soluble in acetone, ether and benzene. When very thoroughly dried nettle leaves are treated with pure acetone, no green colour is extracted, but if a few drops of water are added, the extract becomes green Also if acetone is poured on to fresh leaves, the pigment is extracted The explanation offered for these phenomena is that chlorophyll is present in a colloidal condition in the cell. This point will be considered again later (see p 35)

The Common Nettle (*Urtica*) is the plant which has been used for material for the extraction of chlorophyll on a large scale, and it also forms very useful material for extraction on a small scale The pigment has been found to be unaltered by drying, and, since dried leaves involve far less bulk and dilution of solvents, material should be dried before using Some leaves (Elder and Conifers) are spoilt by drying From dried leaves pure solvents, such as petrol ether, benzene and acetone, extract very little pigment for reasons which will be mentioned later, but if the solvents contain a moderate amount of water, the pigment is readily soluble About 80 % acetone is the best solvent The nettle leaves are removed from the stalks and laid on sheets of paper to dry When well air-dried they are finely powdered, and the powder further dried at 30–40° C in an incubator The leaf-powder can be kept for a considerable time in a well-stoppered bottle.

Expt 18 *Extraction of pigment* Two grams of leaf-powder are sucked to a filter-paper on a small porcelain funnel and 2–3 c.c of 85 % acetone are added. This is allowed to soak into the powder for a few minutes The fluid is then sucked through with the pump. The operation is repeated until 20 c c of the solvent have been added, when the powder is sucked dry A deep blue-green solution with a red fluorescence is obtained which contains all the four pigments from the leaf. The acetone extract thus obtained is then poured into double the quantity of *petrol ether* contained in a separating funnel An equal quantity of distilled water is added, this

being poured gently down the side of the funnel in order to avoid the formation of emulsions In the course of a few minutes, the ether layer separates out and now contains the pigments The lower layer, which is slightly green, is run off The addition of distilled water and subsequent removal of the lower layer is repeated about four times, in order completely to remove the acetone from the ether solution If the ether solution should have become at all emulsified, it can be cleared by shaking with anhydrous sodium sulphate and filtering

The whole process should be repeated with another 2 gms of leaf-powder and the pigment transferred to *ether*, since a solution in this solvent is required for later experiments

Expt 19 *Demonstration of the presence of chlorophylls a and b.* Of the petrol ether solution from the last experiment, 10 c c are shaken with 10 c c of 92 % methyl alcohol Two layers are formed of which the petrol ether layer contains chlorophyll *a*, and the methyl alcohol layer chlorophyll *b* The solution of chlorophyll *a* is blue-green, while that of chlorophyll *b* is a pure green, but the colour difference between them is diminished owing to the presence of the yellow pigments, of which carotin is in the petrol ether, and xanthophyll in the methyl alcohol

As will be explained later, the green pigments of chlorophyll can be saponified by alkalis and are then insoluble in ethereal solution This method can be adopted to separate the green from the yellow pigments, xanthophyll and carotin

Expt 20 *Separation of green and yellow pigments* Shake 5 c c of an ether solution of the pigments (Expt 18) with 2 c c of 30 % caustic potash in methyl alcohol (obtained by dissolving 30 gms of potassium hydroxide in 100 c c of methyl alcohol [1]) After the green colour has reappeared, slowly add 10 c c of water and then add a little more ether On shaking the test-tube, two layers are produced, of which the lower watery-alkaline one contains the saponified green pigments, while the carotin and xanthophyll are contained in the upper ethereal layer

Expt. 21 *Separation of the two yellow pigments* The ether layer obtained in the last experiment is washed with water in a separating funnel, and evaporated down to 1 c c It is then diluted with 10 c c of petrol ether and next mixed with 10 c.c. 90 % methyl alcohol The methyl alcoholic layer is removed and the petrol ether layer is again treated with methyl alcohol and the methyl alcohol again removed This process is repeated until the methyl alcohol is no longer coloured The methyl alcohol contains the xanthophyll, the petrol ether the carotin

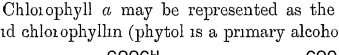
Further accounts of the yellow pigments are given on p. 39

The best known reactions of chlorophyll are those which take place with acids and alkalies respectively.

Chlorophyll is a neutral substance, and, on treatment with alkalies, it forms salts of acids, the latter being known as chlorophyllins These salts are soluble in water forming green solutions which are not however

[1] The methyl alcohol must be very pure, otherwise the alcoholic potash solution will become brown and discoloured

fluorescent Chlorophyll *a* may be represented as the methyl phytyl ester of an acid chlorophyllin (phytol is a primary alcohol, see p 38)



Chlorophyll *a* Chlorophyllin

On treatment in the cold with alkali, the ester is saponified, and the alkali salt of chlorophyllin is formed During saponification, there is a change of colour in the pigment, the so-called brown phase, followed by a return to green

Expt 22 *Saponification of a mixture of the green pigments* Pour a little of the ether solution obtained in Expt 18 into a test-tube, and in a pipette take a little 30 % solution of potash in methyl alcohol Place the lower end of the pipette at the bottom of the test-tube and allow the potash to run in below the chlorophyll solution At the interface between the solutions there appears immediately a brown-coloured layer which diffuses on shaking In about ten minutes it changes back through an olive-green colour to pure green

The chlorophyll has been saponified to the potassium salt of the acid chlorophyllin This salt is insoluble in ether, so if water is added to bring about a separation of the two layers, the green colour is no longer present in the ethereal layer

The change of colour on saponification is different for the two chlorophylls, the brown phase produced in the above mixture of chlorophylls being due to a yellow phase produced by chlorophyll *a*, and a brown-red phase produced by chlorophyll *b* To demonstrate this the phase test (Expt. 22) may also be carried out separately on the two chlorophylls

Expt 23 *Saponification of chlorophylls a and b separately* The methyl alcohol solution obtained in Expt 19 is transferred to ether as in Expt 18 Both the latter and the petrol ether solution of chlorophyll *a* are saponified as in the previous experiment

As already demonstrated the potassium salts of the chlorophyllins which are produced by saponification of the mixture of green pigments in the cold are not fluorescent By saponification of chlorophyll with hot alkali, isochlorophyllins are formed (see Expt 24 below) which are fluorescent

On *heating* chlorophyllins with concentrated alcoholic alkalies, a series of decomposition products, phyllins (also acids) are obtained by removal of carboxyl groups. The final phyllin has only one carboxyl group When this is removed, a substance, aetiophyllin, $C_{31}H_{34}N_4Mg$, is obtained which contains no oxygen (see Scheme 1, p 34).

Another difference between the results of treating chlorophyll with hot and cold alkali is that in the former process the yellow pigments are

destroyed If then water is added after saponification with hot alkali, and the solution is shaken up with ether, the ether will remain colourless

When chlorophyll is treated with acids, a different reaction takes place The chlorophyll changes in colour to olive-green and loses most of its fluorescence The magnesium of the molecule is removed, being replaced by hydrogen, and the resulting product is termed phaeophytin (see Scheme 1, p 34).

From phaeophytin a series of decomposition products have been obtained, which fall into two groups, the phytochlorins and the phytorhodins. The phytochlorins are olive-green in colour, and are derived from chlorophyll *a*, the phytorhodins are red, and are derived from chlorophyll *b* The phaeophytins from the two chlorophylls are indistinguishable until the above decomposition products are obtained. (The original discovery of two kinds of chlorophyll was brought about by the differentiation of these decomposition products)

A number of phytochlorins and phytorhodins have been identified and are designated by letters *a*, *b*, etc By employing a uniform method of treatment, however, two of these products, phytochlorin *e* and phytorhodin *g*, can be secured

The phytochlorins and the phytorhodins are of course magnesium-free compounds and can be obtained by the action of acid on the chlorophyllins and isochlorophyllins Phytochlorin *e* and phytorhodin *g*, in particular are obtained by the action of acid on isochlorophyllins, i.e. they are magnesium-free isochlorophyllins. They are formed by the addition of acid to the products of saponification with hot alkali'

The separation of the various phytochlorins and phytorhodins can be brought about by means of their different distribution between ether and hydrochloric acid· each compound can be extracted from ether according to the concentration of the acid used

Expt 21 *The formation of phytochlorin and phytorhodin.* 5 c c of an ether solution containing both chlorophylls *a* and *b* are evaporated to dryness in a test-tube in a water-bath, and the residue treated with 3 c c of boiling 30 % potash solution in methyl alcohol, and boiled gently for half a minute A liquid with a red fluorescence is produced which consists of a solution of the potassium salts of isochlorophyllins. The solution is diluted with double its volume of water, and concentrated hydrochloric acid is added until the solution is just acid. The liquid is then shaken with ether in a separating funnel . the dissociation products produced by the previous treatment pass into the ether solution which thus acquires an olive-brown colour.

The ether solution is shaken twice, each time with 10 c c. of 4 % hydrochloric acid (sp. gr 1 02 i.e 12 9 c.c. strong acid: 87·1 c c. water), and the green-blue acid layer is separated and neutralized with ammonia and shaken with more ether, which

then contains in solution phytochlorin *e*, the derivative of chlorophyll *a*. The phytochlorin *e* gives to the ether an olive-green colour.

The ether layer remaining in the funnel, after the separation of the green-blue acid layer, is now extracted with 10 c.c. of 12% hydrochloric acid (sp. gr. 1·06 i.e. 38·1 c.c. strong acid + 61·9 c.c. water). The green acid solution so obtained is diluted with water and shaken with ether which then becomes coloured red and contains phytorhodin *g*, the derivative of chlorophyll *b*.

If the phyllins are acted upon by mineral acids, they lose their magnesium in the same way as the chlorophyllins, and the series of substances obtained in this way are termed porphyrins. Thus aetiophyllin will give aetioporphyrin $C_{31}H_{36}N_4$ (see Scheme I, p. 34).

The derivatives of chlorophyll which are free from magnesium, such as phaeophytin, phytochlorin, phytorhodin, the various porphyrins, etc. combine readily with the acetates of some metals such as copper, zinc and iron, and they form intensely coloured, stable compounds. The change of colour is so noticeable that the smallest traces of certain metals can be detected in this way. Hence it is very difficult to prepare the magnesium-free chlorophyll unless the reagents are perfectly pure and all contact with certain metals is avoided.

Expt. 25. *Substitution of copper for magnesium in chlorophyll.* 2 c.c. of an ether solution of chlorophyll are shaken with a little 20% hydrochloric acid (sp. gr. 1·10 i.e. 62·4 c.c. strong acid + 37·6 c.c. water), and then washed with water in a separating funnel. In this way is produced in ether solution the magnesium-free chlorophyll derivative, phaeophytin. The solution is evaporated down on a water-bath, and the residue dissolved in 5 c.c. of alcohol. The solution is olive-green in colour. This is heated and a grain of copper acetate or zinc acetate is added. The colour changes back to a brilliant green, but without fluorescence (if all the chlorophyll has been converted into phaeophytin).

From the results of these recent investigations, it is now possible to write formulae for the two chlorophylls as follows:

chlorophyll *a* $(C_{32}H_{30}O\,N_4Mg)\,(COOCH_3)\,(COOC_{20}H_{39})$
chlorophyll *b* $(C_{32}H_{28}O_2N_4Mg)\,(COOCH_3)\,(COOC_{20}H_{39})$

from which it will be seen that the phytol component amounts to one-third of the weight of the chlorophyll.

From the analyses of chlorophylls from different plants, it was found that the phytol content varied, and plants which yielded little phytol most readily produced "crystalline chlorophyll," a form of the pigment which has been known for some considerable time to previous workers. The Cow Parsnip (*Heracleum Sphondylium*), Hedge Woundwort (*Stachys sylvatica*) and Hemp-nettle (*Galeopsis Tetrahit*) are plants which readily give crystalline chlorophyll. In this connexion it has been suggested

that the chlorophyll in plants is accompanied by an enzyme, chlorophyllase, which, in alcoholic media, brings about alcoholysis of the chlorophyll, and replaces the phytyl by the ethyl radicle The products, formerly known as crystalline chlorophyll, are now termed chlorophyllides

$$(C_{32}H_{30}ON_4Mg)(COOCH_3)(COOC_{20}H_{39}) + C_2H_5OH = C_{20}H_{39}OH + (C_{32}H_{30}ON_4Mg)(COOCH_3)(COOC_2H_5)$$

Phytol — Ethyl chlorophyllide

Similar chlorophyllides are produced by other alcohols In aqueous solutions chlorophyllase brings about hydrolysis and the free acid chlorophyllide is formed (see Scheme 2, p 34)·

$$(C_{32}H_{30}ON_4Mg)(COOCH_3)(COOC_{20}H_{39}) + H_2O = C_{20}H_{39}OH + (C_{32}H_{30}ON_4Mg)(COOCH_3)(COOH)$$

Chlorophyllide

Chlorophyllase is a very stable enzyme , it is not even destroyed by boiling in alcohol for a short time, but if leaves are boiled in water, the enzyme is destroyed

Expt 26 *Microscopic examination of ethyl chlorophyllide* Prepare sections of fresh *Heracleum* leaves and mount them in a drop of 90 % alcohol. Leave the slide under a bell-jar containing a dish of alcohol The section slowly dries in the course of half a day or a day It is then examined under the microscope when there will be observed the characteristic triangular and hexagonal crystals of ethyl chlorophyllide (crystalline chlorophyll)

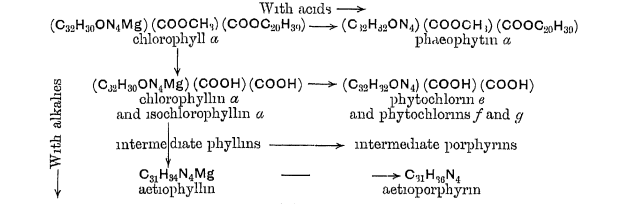
Expt 27 *Production of methyl chlorophyllide in the leaf* Sections may be used as in the preceding experiment, or a piece of a leaf may be employed In the latter case a test-tube with 4 c c of 75 % methyl alcohol is taken and 1 gm of fresh leaf is added to it The leaf first becomes a darker green and then during the course of a few hours becomes yellowish. On holding the leaf to the light there can be observed with the naked eye a number of black points If sections of the leaf be cut and examined under the microscope, these spots appear as aggregates composed of rhombohedral crystals, occurring in certain cells

Expt 28 *Extraction of ethyl chlorophyllide* Two grams of dry *Heracleum* leaf-powder are left for a day in a test-tube containing 6 c c of 90 % alcohol The extract is then filtered through a small porcelain funnel and the powder on the filter washed with a little acetone The filtrate is mixed with an equal quantity of ether, and then with some water The ether solution is transferred to a separating funnel and thoroughly washed with water, and then concentrated on a water-bath to ½ or 1 c c , and 3 c.c of petrol ether are added. On standing, the ethyl chlorophyllide is precipitated in the form of crystalline aggregates. It is freed from yellow pigments by shaking with a *little* ether, and can be further purified by redissolving in ether and precipitating again with petrol ether.

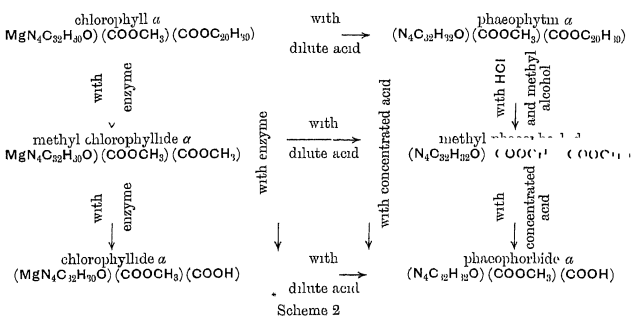
Expt 29. *The action of chlorophyllase* Fresh leaves of a species rich in chlorophyllase, e g. *Heracleum* or *Galeopsis*, are finely divided and put in a 70 % acetone solution, 3 c c of solution being used for every gram of leaf-powder. The chlorophyll,

by means of the chlorophyllase, is hydrolyzed into phytol and the acid chlorophyllide This can be demonstrated after about a quarter of an hour if the solution is diluted with water, transferred to ether and shaken with 0 05 % sodium hydroxide The sodium hydroxide takes up more colouring matter the further the enzyme action has progressed

Expt 30 The destruction of chlorophyllase If fresh leaves of a species rich in chlorophyllase are first steeped in boiling water for a few minutes before they are placed in the acetone solution, unaltered chlorophyll is extracted which does not react with dilute alkali



Scheme 1



Scheme 2

By treatment with acids, magnesium is removed from the chlorophyllides, with the production of the corresponding phaeophorbides. Thus methyl chlorophyllide *a* gives methyl phaeophorbide *a*, etc. (see Scheme 2, above)

It has been previously mentioned that water-free solvents, such as acetone, ether and benzene, in which pure extracted chlorophyll is

soluble, will not extract the pigment from thoroughly dried leaves, but if a little water is added, it readily goes into solution From fresh leaves also these solvents can extract the pigments

As an explanation of the above phenomena, it has been suggested that chlorophyll in the chloroplastid is in the colloidal state, and that, when water is added to the dried leaf, a solution of mineral salts in the leaf is formed which alters the colloidal condition of the chlorophyll and makes it soluble. This view is supported by the fact that if a colloidal solution of chlorophyll in water, made from the pure extracted pigment, is shaken with ether, the ether remains colourless If, however, a little salt solution is added and the mixture shaken, the ethereal layer becomes green In preparing the colloidal solution the solvent, acetone, is replaced by the medium, water, in which chlorophyll is insoluble

The condition of chlorophyll is altered by plunging the leaves into boiling water The pigment is then much more readily soluble in ether, etc., even when the leaves are subsequently dried It is supposed that the chlorophyll has diffused out from the plastids, and is in true solution in accompanying waxy substances which have become liquid owing to change of temperature.

Expt 31 *Preparation of a colloidal solution of chlorophyll* Take 10 c c of an acetone extract of chlorophyll (Expt. 18) and pour this acetone solution into a large volume of distilled water (100 c c.), the liquid being continually stirred This operation can be most conveniently done by taking the acetone solution in a pipette and allowing it to run out of the pipette while the latter is used as a stirring rod in the water. Note the change in colour to a purer green, and the disappearance of fluorescence

Expt 32 *To demonstrate the difference between a true and a colloidal solution of chlorophyll* Evaporate 10 c c of an acetone extract (Expt. 18) to complete dryness and test its solubility in ether, petrol ether and benzene It is soluble in all three solvents Now add these solvents to some of the colloidal solution prepared in the last experiment, and note that the chlorophyll does not dissolve in any of these solvents If, however, some salt solution, e.g. a little magnesium sulphate, be added, the chlorophyll is precipitated from its colloidal state and is now soluble in ether and other solvents.

Expt 33. *To show that chlorophyll in the plant is probably in the colloidal condition* Some nettle powder is carefully dried, e g. by keeping it at 30–40° C. in an oven, and then further drying in a vacuum desiccator over sulphuric acid. Small quantities of this dry powder are put in test-tubes, and different pure water-free substances such as acetone, ether, benzene and absolute alcohol are added Note that these solvents are not coloured by the chlorophyll. It can be demonstrated that the extracted pigment is easily soluble in any of these substances Repeat the experiment with nettle powder moistened with a few drops of water, and note that the solvents are immediately coloured.

Expt 34 *Pure solvents are able to extract chlorophyll from fresh leaves* Crush 10 gms of fresh leaves of nettle, horse-chestnut or elder in a mortar with some clean sand, and put the crushed material on a filter-paper in a porcelain funnel Add 20 c c of pure acetone and suck it through by means of a water-pump Repeat this several times The pure solvent is here able to extract the pigment

Expt 35 *Treatment of fresh leaves with boiling water changes the condition of the chlorophyll* Dry a quantity of leaves which have been put in boiling water and examine their solubility as in Expt 33 Note that the chlorophyll in this powder is soluble in pure solvents

There is finally another change which chlorophyll can undergo, namely that of allomerization, which takes place in alcoholic solution The characteristic of allomerized chlorophyll is that it does not give the brown phase when treated with alkali (see Expt 22) Allomerization is accelerated in alkaline solution but inhibited by small quantities of acid

Expt 36 *To demonstrate that allomerized chlorophyll does not give the brown phase test* Dissolve a little crude chlorophyll, obtained by evaporating an ether solution, in absolute alcohol To a sample of this add a little alkali, and perform the phase test, from time to time, till at last the brown phase no longer appears

Connexion of Chlorophyll with Formaldehyde

In addition to the above, another chemical property of chlorophyll of great interest, is that connected with the production of formaldehyde Those investigators, who have sought to confirm the formaldehyde hypothesis of carbon assimilation, have based their evidence on tests for formaldehyde both in the plant and in chlorophyll-containing systems outside the plant By exposing films, or solutions, of chlorophyll to light in presence of carbon dioxide, they have detected formaldehyde as a result (Usher and Priestley, 5)

The most recent investigations (Jorgensen and Kidd, 2) have shown that the experimental evidence is at present inadequate to support the hypothesis, since formaldehyde arises from chlorophyll itself in the absence of carbon dioxide

In this later work (Jorgensen and Kidd, 2) on the behaviour of extracted chlorophyll in light, use has been made of a colloidal solution (see p 35) of pure chlorophyll (chlorophylls *a* and *b*) for experimental work The solution for this purpose must be prepared from pure chlorophyll, which has been tested and shown to be free from yellow pigments, since the latter absorb oxygen and may confuse the issue of the experiment. The pure chlorophyll is prepared by extracting dried nettle leaves with 80–85 % acetone in the usual way and transferring to petrol ether (p 28).

The petrol ether extract is then washed with 80 % acetone to remove colourless impurities, and with 80 % methyl alcohol to remove xanthophyll Finally all traces of acetone and methyl alcohol are removed by washing with water This renders the chlorophyll insoluble in petrol ether, since it is only soluble in this solvent if traces of other solvents are present Hence the pigment is precipitated out as a fine suspension, leaving the carotin in solution. The chlorophyll is filtered off through powdered talc, taken up in ether, reprecipitated by petrol ether and finally obtained as a blue-black micro-crystalline substance The colloidal solution or sol is made by dissolving 0·4 gm of pure chlorophyll in 3 c c of absolute alcohol and pouring into 300 c c of distilled water

The advantage of using such a solution is that the experimental conditions, in all probability, approach more nearly to the conditions in the plant, and reactions with other substances take place more readily than when the chlorophyll is used as a film The use of pure, instead of crude, chlorophyll is also important as by this means it is possible to determine the changes taking place in chlorophyll itself without complications arising from the accompanying impurities The discordant results of various workers on this subject are doubtless due to the employment of crude chlorophyll Ethyl alcohol is the best solvent for preparing the sol since it does not produce formaldehyde when exposed to light under ordinary circumstances in glass vessels Methyl alcohol and acetone should be avoided as they themselves either contain or give rise to formaldehyde

The chlorophyll sol is electro-negative It is stabilized by weak alkalies, but precipitated by weak acids

Working with such a colloidal solution the results may be summarized as follows

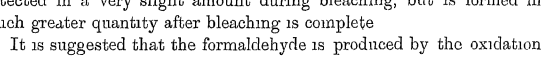
When a chlorophyll sol is exposed to light in an atmosphere of nitrogen in a sealed tube, no apparent change takes place in the chlorophyll, and no formaldehyde is produced

When exposed in an atmosphere of carbon dioxide in a sealed tube, the chlorophyll rapidly turns yellow- or brown-green In the case of sols of high concentration, the colour-change is preceded by precipitation of the pigment. The same change takes place in the dark, only more slowly. No formaldehyde is produced, and no absorption of carbon dioxide could be detected. The yellow product has been shown to be the magnesium-free derivative, phæophytin, which is produced from the pigment by the action of acids. The changes observed are explained by the fact that the carbon dioxide, acting as a weak acid, first precipitates

the sol, if concentrated, and then acts, like other weak acids, on the chlorophyll, producing phaeophytin If the solution is kept neutral by addition of sodium bicarbonate, there is no colour change The identity of phaeophytin was shown by the spectrum and by the restoration of colour on adding a trace of copper acetate

When exposed to light, and the atmosphere in the sealed tube is replaced by oxygen or air, the chlorophyll turns yellow- or brown-green as before and then bleaches The change of colour from green to yellow or brown is again due to the formation of phaeophytin, this being brought about by the presence of an acid substance, which is produced during bleaching, and increases throughout the process Formaldehyde can be detected in a very slight amount during bleaching, but is formed in much greater quantity after bleaching is complete

It is suggested that the formaldehyde is produced by the oxidation and breaking down of the phytol component of the chlorophyll



There is no reason for ascribing to any of the above reactions any part in carbon assimilation There is at present no hypothesis, supported by satisfactory evidence, as to the process of carbon assimilation

Expt 37 *Detection of formaldehyde as a product of oxidation of chlorophyll* Extract 2 gms of dried nettle leaf powder with 20 c c of 80 % acetone and transfer it to petrol ether as in Expt 18 Then shake the petrol ether extract four or five times with an equal volume of 80 % acetone to remove colourless impurities Next the petrol ether extract is similarly shaken up with 80 % methyl alcohol which removes the xanthophyll This should be repeated until the methyl alcohol is colourless The petrol ether is finally washed repeatedly with water to remove traces of acetone and methyl alcohol The chlorophyll is in time precipitated as a fine suspension, being insoluble in pure petrol ether This suspension is filtered through either kieselguhr or powdered talc on a small porcelain filter The chlorophyll is extracted from the powder on the filter with as small a quantity as possible of absolute alcohol This alcoholic solution is then poured, with constant stirring, into 100 c c of distilled water by which means a colloidal solution of chlorophyll is obtained

The test to be employed for formaldehyde is as follows (Schryver, 4) To 10 c.c of the liquid to be tested add 2 c c of a 1 % solution (freshly made) of phenylhydrazine hydrochloride, 1 c c of a 5 % solution (freshly made) of potassium ferricyanide and 5 c c of concentrated hydrochloric acid If formaldehyde is present a pink to magenta colour is developed, either deep or pale, according to the quantity of formaldehyde The reaction is due to the formation of a condensation product of formaldehyde and phenylhydrazine, and this compound, on oxidation, yields a weak base forming a coloured salt with concentrated hydrochloric acid The salt is readily dissociated again on dilution of the solution

Two modifications (Schryver, 4) can be adopted in applying this test First, in testing for formaldehyde in pigmented solutions, the following course can be pursued The reaction mixture, after addition of phenylhydrazine, ferricyanide and hydrochloric acid, is diluted with water, and ether is added in a separating funnel The hydrochloride of the chromatogenic base is dissociated and the base is taken up by the ether The aqueous solution is run off, and on addition of strong hydrochloric acid to the ether, the base passes into the acid as a coloured hydrochloride again By using a small quantity of acid, the sensitiveness of the test is increased, since the colour is now distributed through a small quantity of liquid only

The second modification consists in warming the solution to be tested for a short time with the phenylhydrazine hydrochloride before adding the other reagents In this way, formaldehyde can also be detected if it should be in a polymerized form

As a control, 10 c c of the colloidal solution of chlorophyll should be tested, using both the above modifications The remainder of the solution should be exposed to sunlight (or the light from either an arc or mercury vapour lamp) in a loosely corked vessel, until it is completely bleached The bleached solution, on testing, will be found to give a positive test for formaldehyde

The Yellow Plastid Pigments

These have already been mentioned in connexion with the leaf pigments (pp 28 and 29) In addition, however, they have a further significance in that they constitute the pigments, located in plastids, of most yellow and orange flowers and fruits Sometimes also they occur in other organs, i e root of Carrot (carotin).

Carotin, $C_{10}H_{56}$, is an unsaturated hydrocarbon It crystallizes in lustrous rhombohedra which are orange-red by transmitted and blue by reflected light. It is readily soluble in chloroform, benzene and carbon bisulphide, but with difficulty in petrol ether and ether

One of its most characteristic properties is that it readily undergoes oxidation in air, and becomes bleached With concentrated sulphuric acid it gives a deep blue colour.

Xanthophyll, $C_{40}H_{56}O_2$, also forms yellow crystals with a blue lustre. It is soluble in chloroform and ether, but insoluble in petrol ether. It is more soluble than carotin in methyl alcohol It gives a blue colour with sulphuric acid, and also oxidizes in air with bleaching

The separation of the two pigments (see Expt 21) is based on the fact that in a mixture of petrol ether and methyl alcohol containing a little water, the carotin passes entirely into the petrol ether, whereas the greater part of the xanthophyll remains in the methyl alcohol layer.

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Books

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Papers

2 **Jorgensen, I.**, and **Kidd, F.** Some Photochemical Experiments with Pure Chlorophyll and their Bearing on Theories of Carbon Assimilation. *Proc R Soc*, 1917, B Vol 89, pp 342–361

3 **Jorgensen, I.**, and **Stiles, W.** Carbon Assimilation A Review of Recent Work on the Pigments of the Green Leaf and the Processes connected with them *New Phytologist*, Reprint, No 10 London, 1917

4 **Schryver, S. B.** The Photochemical Formation of Formaldehyde in Green Plants *Proc R Soc*, 1910, B Vol 82, pp 226–232

5 **Usher, F. L**, and **Priestley, J. H.** A Study of the Mechanism of Carbon Assimilation in Green Plants I *Proc R Soc*, 1906, B Vol 77, pp 369–376 II *Ibid* 1906, B Vol 78, pp 318–327 III *Ibid* 1912, B Vol 84, pp 101–112

CHAPTER V

CARBOHYDRATES AND THEIR HYDROLYZING ENZYMES

THE carbohydrates which occur in plants may be classified as follows

Monosaccharides	Pentoses, $C_5H_{10}O_5$—Arabinose, xylose Hexoses, $C_6H_{12}O_6$—Glucose, galactose, mannose, laevulose
Disaccharides ..	Sucrose, maltose, $C_{12}H_{22}O_{11}$.
Trisaccharides	Raffinose and others.
Polysaccharides ..	Pentosans, $(C_5H_8O_4)_n$—Araban, xylan Starches, $(C_6H_{10}O_5)_n$—Starch, dextrin, inulin Mannans, galactans, gums, mucilages, pectic substances Celluloses, $(C_6H_{10}O_5)_n$

The carbohydrates are widely distributed in plants and form most important parts of their structure Those most commonly found are cellulose, starch, pentosans, dextrin, glucose, sucrose, laevulose, and maltose Other sugars, especially trisaccharides, are known in addition to those mentioned above, but they are somewhat restricted and specific in their distribution

As in the case of the proteins, so with the carbohydrates, the molecules of the more simple and soluble crystalline compounds, such as the monosaccharides, are synthesized into more complex molecules which exist, either in the colloidal (dextrin), or insoluble state (starch, cellulose) The last-mentioned build up parts of the solid structure of the plant The resolution of the solid complex substances into simple ones is known in many instances to be brought about in the plant by enzymes, and it is highly probable that the synthesis of the complex from the simple is also controlled by these enzymes

The most commonly occurring sugars in plants are glucose, laevulose, sucrose and maltose sucrose is hydrolyzed by the enzyme, invertase, into one molecule of glucose and one molecule of laevulose · maltose by the enzyme, maltase, into two molecules of glucose Both invertase and maltase are widely distributed. The connexion between various sugars and photosynthesis, and their inter-relationships with each other in the leaves, are reserved for another section.

Of the polysaccharides, cellulose is universally distributed in higher plants and constitutes the greater part of the cell-walls The pentosans, galactans and mannans also, but to a lesser degree, are components of their structure Starch, in addition, is very widely distributed it is converted by the enzyme, diastase, into dextrin and maltose, and possibly the same enzyme also controls its synthesis In some plants no starch is formed, and its place in metabolism is taken by inulin or cane-sugar

The various carbohydrates will first be dealt with in detail, and later their inter-relationships will be considered

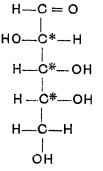
Monosaccharides

These are termed tetroses, pentoses or hexoses according to the number of carbon atoms in the molecule. They contain primary ($-CH_2OH$) or secondary ($=CHOH$) alcohol groups, and either an aldehyde ($-CHO$) group, as in glucose, or a ketone ($=C=O$) group, as in laevulose They are, as a class, white crystalline substances, soluble in water and aqueous alcohol, but insoluble in ether, acetone and many other organic solvents They are capable of certain characteristic chemical reactions which form a basis for their detection and estimation. One of the most important is that connected with the aldehyde and ketone groups, owing to which they act as reducing agents, being themselves oxidized The reducing action usually employed is that which takes place with copper salts in hot alkaline solution, whereby cuprous oxide is formed. Hence they are termed "reducing" sugars. Another important reaction is the formation of crystalline osazones (only in the case of sugars with aldehyde or ketone groups), which, by virtue of their melting points and characteristic crystalline forms, constitute, in several cases, valuable tests for the presence of sugars

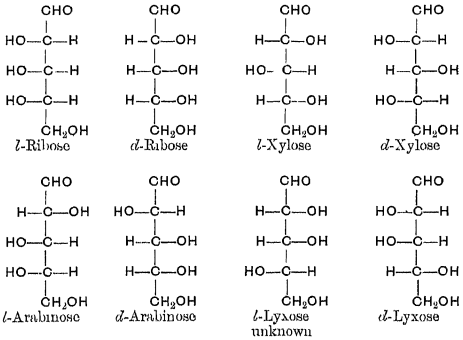
Pentoses.

These sugars contain five carbon atoms, and have the general formula $C_5H_{10}O_5$. They are said to be present in the free state to some extent in leaves (Davis and Sawyer, 10) In plants they occur chiefly, however, as condensation products formed with elimination of water. These products are termed the pentosans, and are widely distributed, on hydrolysis they yield pentoses again The various gums found in plants consist largely of pentosans, and the pectins also contain pentose groups, both consequently yield pentoses on hydrolysis (see pp 61 and 64).

If we examine the structural formula of a pentose, as for example, arabinose



we see that each of the three carbon atoms marked * is united to four different atoms or groups of atoms Each of these carbon atoms is therefore asymmetric, and, with regard to it, there are two possible isomers (see p 9, Cole, 3, for stereoisomerism) It will be found on examination that there are eight possible isomers of the formulae given above



Of these only seven have been isolated. The two pentoses which occur in plants are *l*-arabinose and *l*-xylose These, however, are known almost solely as condensation products, pentosans, in gums, woody tissue, etc The pentoses form osazones (see p 49 for reactions and composition).

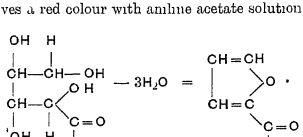
Arabinose. This sugar occurs as the pentosan, araban, in various gums, such as Cherry Gum, Gum Arabic, etc (see p 45).

Some of the properties and reactions of the pentoses are demonstrated in the following experiments

Expt 38 *Tests for arabinose* For reactions *a–e* use a 1 % solution of arabinose for reaction *f* a 0 2 % solution

If pure arabinose is not available, a solution for tests *a*, *b* and *c* can be prepared from gum arabic Boil 5 gms of the gum in 100 c c of water with 10 c c of concentrated hydrochloric acid for 5 minutes and then neutralize to litmus with alkali Such a solution is only suitable for the specific tests for arabinose, since it also contains galactose (see p 61) For tests *a*, *b* and *c* small pieces of solid gum arabic may even be used

(*a*) Heat a few c c of the sugar solution in a test-tube with about half its volume of concentrated hydrochloric acid In the mouth of the test-tube place a piece of filter-paper soaked with aniline acetate (made by mixing equal quantities of aniline, water and glacial acetic acid) A pink colour will be produced in the paper This is due to the fact that furfural is formed by the action of the acid on the pentose, and the furfural then gives a red colour with aniline acetate solution



Arabinose Furfural

This reaction, however, is also given by the hexoses but to a much less extent

(*b*) Warm a few c c of the sugar solution with an equal volume of concentrated hydrochloric acid in a test-tube, and add a small quantity of phloroglucin A bright red coloration is produced

(*c*) To a few c c of the sugar solution in a test-tube add an equal quantity of concentrated hydrochloric acid, and then a little solid orcinol Divide the solution into two equal portions. Heat one portion The solution will become red changing to violet and finally blue, blue-green or green To the other portion, after heating for a time, add a few drops of 10 % ferric chloride solution A deep green colour is at once produced In both cases, on the addition of a little amyl alcohol, the green colour will be extracted by the alcohol

(*d*) *a*-Naphthol reaction Add to a little of the sugar solution a few drops of a 1 % solution of *a*-naphthol in alcohol Mix the two solutions and then run in about 5 c c of concentrated sulphuric acid down the side of the test-tube A violet coloration is produced at the junction of the two liquids. The coloration is due to a condensation product of *a*-naphthol with furfural, the latter being formed by the action of the acid on the carbohydrate This reaction is likewise given by laevulose and cane-sugar (since it yields laevulose, see p 52), and less strongly by glucose and maltose, also by some proteins which contain a carbohydrate group

(*e*) Boil a little of the arabinose solution with a few drops of Fehling's solution Reduction will take place

(*f*) Make the osazone of arabinose following the instructions given for glucosazone (see p 49)

A solution of arabinose which will give the pentose reactions can also be obtained by hydrolysis of Cherry Gum The gum oozes from the bark of various species of *Prunus*, such as the Cherry (*Prunus Cerasus*) and the Bird Cherry (*P Padus*)

Expt 39 *Preparation of an abunose solution from Cherry Gum* The gum is heated, on a water-bath in a round-bottomed flask fitted with an air condenser[1], with dilute sulphuric acid (1 pt by wt of gum 7 pts by wt of 4 % sulphuric acid) for about 5 hours The solution is then neutralized with calcium carbonate and filtered Perform the tests *a*, *b* and *c* of Expt 38 on the solution A positive result is obtained in each case Since the solution contains other sugars as impurities, it cannot conclusively be used for tests *d*, *e* and *f* If a considerable quantity of gum is available, crystallization of arabinose should be attempted by concentrating the aqueous sugar solution, extracting this with 90 % alcohol and again concentrating in a desiccator (see p 53) If a very small quantity of gum only is available, the tests *a*, *b* and *c* should be performed directly on a small piece of the gum in a test-tube

A purer preparation of arabinose, which may be used for all the tests of Expt 38, can be obtained by the hydrolysis of araban (see Expt 48)

Xylose This sugar occurs very widely distributed in woody tissue as the pentosan, xylan (see p 53). A solution of xylose which will give the pentose reactions can be obtained from the hydrolysis of straw

Expt 40 *Preparation of xylose solution from straw* Take about 50 gms of straw, which has been cut up into small pieces, and put it into a round-bottomed flask fitted with an air condenser Add sufficient 5 % sulphuric acid to cover the straw and heat on a water-bath for 2–3 hrs Filter off the solution, neutralize with calcium carbonate and filter again Make with the solution the tests *a* and *c* of Expt 38 The solution will also reduce Fehling's solution strongly, but this reduction may be partly due to other sugars formed in the hydrolysis

The presence of xylan giving the pentose reactions can also be demonstrated in straw, bran or sawdust by merely heating small quantities of these substances in a test-tube with the above reagents (see Expt 49)

A purer solution of xylose can be obtained from the hydrolysis of xylan (see Expt 51).

When xylose is oxidized with bromine, it yields xylonic acid which has a characteristic cadmium salt. The formation of this salt is used as a method for identifying the sugar (see Expt 51)

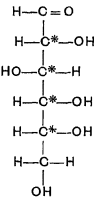
Hexoses.

Glucose. This substance, which is also known as grape-sugar, is very common and very widely distributed in plants. It occurs in the tissues of leaves, stems, roots, flowers and fruits It is produced as a result of

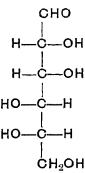
[1] i e a wide piece of glass tubing about 3 ft long passing through the cork

the hydrolysis of cane-sugar and maltose, and, in all probability, is the first sugar synthesized from carbon dioxide and water Its synthesis and its relationships to other sugars will be discussed later (see p 69). It is a white crystalline substance, readily soluble in water and aqueous alcohol, but only slightly soluble in absolute alcohol

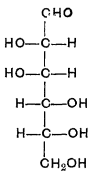
If, as in the case of a pentose, we examine the structural formula for a hexose, such as glucose



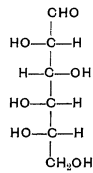
we see that there are four carbon atoms marked * which are united to four different groups of atoms It will be found in this case that there are sixteen possible isomers



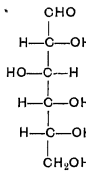
l-Mannose



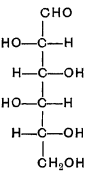
d-Mannose



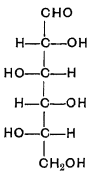
l-Glucose



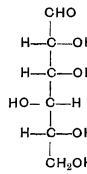
d-Glucose



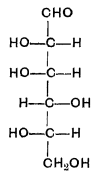
l-Idose



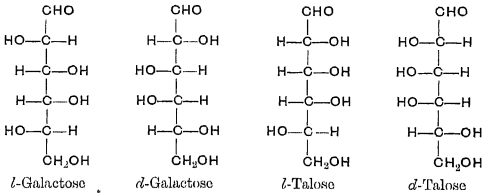
d-Idose

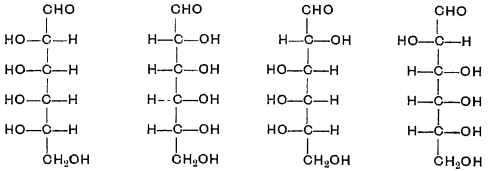


l-Gulose



d-Gulose





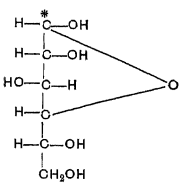
These four unknown

Though many of the above sugars have been synthesized artificially, only three are known to occur naturally, i e *d*-glucose (dextrose or grape-sugar), *d*-mannose and *d*-galactose

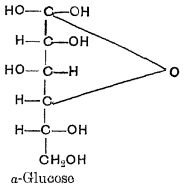
Since compounds containing asymmetric carbon atoms are optically active, i.e can rotate a plane of polarized light, it follows that the sugars under discussion are optically active

d-glucose is dextro-rotatory

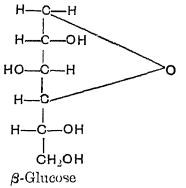
When either *d*- or *l*-glucose is first dissolved in water, it is chemically less active than would be expected of the aldehyde form depicted above. This is explained by assuming that glucose, when first dissolved in water, exists in the condition of a γ-lactone



In the above state the carbon atom marked * is also asymmetric so hat two forms of glucose are possible, α- and β-glucose



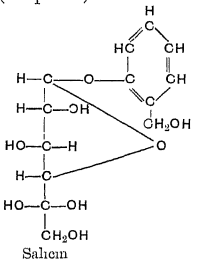
α-Glucose



β-Glucose

In solution, both the above forms pass by tautomerism into the dehyde form

In the plant there are, as will be described later (p 142), many aromatic nd other compounds containing one or more hydroxyl groups These ydroxyl groups of the aromatic substances are frequently replaced by glucose (or other sugar) molecule, and such compounds are termed lucosides, as, for instance, salicin, the glucoside of salicylic alcohol which ccurs in Willow bark (see p 152)



Salicin

These substances, moreover, may be classified either as α- or β-ucosides according to which of the above α or β forms of glucose mbine with the residual part of the compound Various glucosides will e dealt with in Chaps VII and IX.

Expt 41 *Tests for glucose.* Before dealing with the sugars actually isolated from e plant, it is advisable that the following tests and reactions should be performed th pure glucose using a 0 2% solution.

(*a*) *Moore's test* Boil a little of the glucose solution with an equal volume of ustic soda solution A yellow colour is developed which is due to the formation of ondensation product (caramel) of the sugar.

(*b*) *Trommer's test* Add a few drops of a 1 % copper sulphate solution to 2–3 c c of 5 % caustic soda solution A blue precipitate of cupric hydroxide is formed Add now 2–3 c c of the glucose solution, and the precipitate will dissolve On boiling, the blue colour disappears, and a yellow or red precipitate of cuprous oxide is formed If only a little sugar is present the blue colour will disappear, but no oxide may be formed

(*c*) *Fehling's test* Boil a few c c of freshly made Fehling's solution in a test-tube and note that it is unaltered Then add an equal quantity of the glucose solution and boil again A red precipitate of cuprous oxide is formed

(*d*) *Osazone test* Take 10 c c of a 0 5 % solution of glucose in a test-tube and add as much solid phenylhydrazine hydrochloride as will lie on a sixpenny piece, at least twice as much solid sodium acetate and also 1 c c of strong acetic acid Warm gently until the mixture is dissolved and filter into another test-tube Then place the tube in a beaker of boiling water for at least ½ hour, keeping the water boiling all the time Let the test-tube cool slowly, and a yellow crystalline deposit of phenylglucosazone will separate out Examine this under the microscope and it will be found to consist of fine yellow needles variously aggregated into sheaves and rosettes Glucosazone melts at 204–205° C

The osazone reaction takes place as follows

$$CH_2OH(CHOH)_4CHO + H_2N \cdot NHC_6H_5 = \underset{\text{Glucose phenylhydrazone}}{CH_2OH(CHOH)_4CH \quad N \quad NHC_6H_5} + H_2O$$

The phenylhydrazone is very soluble, but if an excess of phenylhydrazine is used, a second hydrazine complex is introduced and an insoluble osazone is formed

$$CH_2OH(CHOH)_3—\underset{\underset{N \cdot NHC_6H_5}{\|}}{C}—CH \quad N \quad NHC_6H_5$$

Glucose reacts in this way by virtue of its aldehyde group Phenylhydrazine hydrochloride does not give an osazone when boiled with glucose unless excess of sodium acetate be added This acts on the hydrochloride to form phenylhydrazine acetate and sodium chloride

Galactose. Galactose rarely, if ever, occurs free in plants, though it is fairly widely distributed in the form of condensation products, the galactans, in combination with other hexoses and with pentoses (see p 60). These galactans form constituents of various gums, mucilages, etc. Agar-agar, which is a mucilage obtained from certain genera of the Red Seaweeds (Rhodophyceae), yields a high percentage of galactose on hydrolysis with acids Galactose also occurs as a constituent of some glucosides from which it may be derived on hydrolysis.

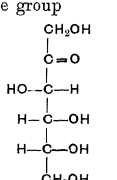
One of the most important reactions of galactose is the formation of mucic acid on oxidation with nitric acid. Mucic acid is practically insoluble in water and separates out as a crystalline precipitate on pouring the products of oxidation into excess of water

Expt 42 *Preparation of galactose from agar-agar* Weigh out 50 gms of agar-agar Put it into a round-bottomed flask fitted with an air condenser (see p 45) Add 500 c c of 2 % sulphuric acid and heat on a water-bath for 4 hrs Neutralize the solution with calcium carbonate and filter Concentrate on a water-bath to a syrup On standing, crystals of galactose will separate out Then add a little 50–75% alcohol and warm gently on a water-bath By this means much of the dark-coloured product will go into solution and can be poured off leaving the crystalline residue Take up this residue in a little hot water, boil well with animal charcoal to decolorize the solution and filter Concentrate again on a water-bath On cooling, colourless prisms of galactose will separate out

Expt 43 *Oxidation of galactose to mucic acid* Heat the galactose obtained in the last experiment with nitric acid (1 gm galactose to 12 c c of nitric acid of sp gr 1 15, i e 5 pts of concentrated acid and 12 pts of water) on a water-bath, until the liquid is reduced to one-third of its bulk Then pour the product into excess of distilled water On standing (for a day or two), a white sandy microcrystalline precipitate of mucic acid will separate out

Mannose. Mannose has not been detected free in many plants, but is widely distributed as condensation products, the mannans, in certain mucilages and in the cell-walls of the endosperm of various seeds (see p 59) From the mannans the sugar can be obtained by hydrolysis On adding phenylhydrazine hydrochloride and sodium acetate to a solution of mannose, the phenylhydrazone, which is nearly insoluble in water, is formed almost immediately and hence constitutes a ready method for the detection of the sugar

Laevulose. This sugar, which is also termed fructose, is widely distributed in plants, in the tissues of leaves, stems, fruits, etc It is formed, together with glucose, in the hydrolysis by acids of cane-sugar. The original cane-sugar is dextro-rotatory, whereas laevulose is more laevo-rotatory than glucose is dextro-rotatory, hence the mixture from the hydrolysis is laevo-rotatory and is known as invert sugar, the change being termed inversion The same hydrolysis is brought about by the widely distributed enzyme, invertase The polysaccharide, inulin, also yields laevulose on acid hydrolysis Laevulose is a white crystalline substance, soluble in water and alcohol Unlike glucose, it contains a ketone instead of an aldehyde group



Laevulose reduces Fehling's and other copper solutions It yields the same osazone as glucose with phenylhydrazine hydrochloride and sodium acetate It also forms an osazone with methylphenylhydrazine (m.p 158° C), a reaction which constitutes a distinction from glucose since the latter gives no osazone with this substance.

Expt 44 *Tests for laevulose* The following tests should be performed with a 0 2% solution of laevulose in the same way as for glucose (see p. 48)

(*a*) *Mooi's test* A positive result is obtained

(*b*) *Trommer's test* A positive result is obtained

(*c*) *Fehling's test* Reduction takes place

(*d*) *Osazone test* Note that the crystals are identical with those formed from glucose

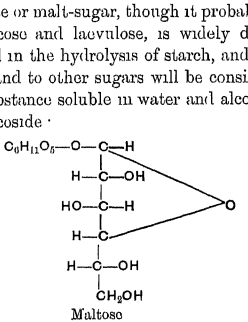
(*e*) *α-Naphthol test* (see p 44) A strong reaction is given

(*f*) *Selwanoff's test* To 5 c c of Selwanoff's solution (prepared by dissolving 0 05 gm of resorcinol in 100 c c of 1 in 2 hydrochloric acid) add a few drops of laevulose solution and boil A red coloration and a red precipitate are formed Add a little alcohol and the precipitate forms a red solution

Disaccharides.

These sugars are formed from the monosaccharides by condensation with elimination of water By boiling with dilute acids, or by the action of certain enzymes, they are hydrolyzed into monosaccharides The two most important disaccharides found in plants are maltose and cane-sugar

Maltose. Maltose or malt-sugar, though it probably occurs in smaller quantities than glucose and laevulose, is widely distributed in plant tissues It is formed in the hydrolysis of starch, and its relationships in the plant to starch and to other sugars will be considered later. It is a white crystalline substance soluble in water and alcohol In constitution it is a glucose-α-glucoside ·



Maltose

It reduces Fehling's solution, but less readily than glucose. With phenylhydrazine hydrochloride and sodium acetate it forms an osazone

(m p 206° C), which is more soluble than glucosazone and crystallizes in broader flatter needles Maltose is dextro-rotatory.

Expt 45 *Tests for maltose* The tests *a*, *b*, *c* and *e* should be performed with a 0 2 % solution of maltose, test *d* with a 2 % solution (see also glucose, p 48)

(*a*) *Mooré's test* A positive reaction is given

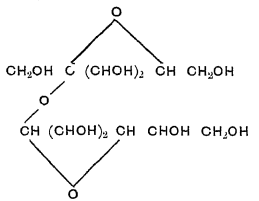
(*b*) *Trommer's test* A positive reaction is given

(*c*) *Fehling's test* Reduction takes place, but less strongly than with glucose

(*d*) *Osazone test* Take 10 c c of the solution and treat as for glucosazone The crystals of maltosazone will be found to be much broader than those of glucosazone

(*e*) *Hydrolysis* Take 20 c c of the sugar solution and add 2 c c of concentrated hydrochloric acid Heat in a boiling water-bath for half an hour Neutralize and test for the osazone Glucosazone will be formed

Sucrose. Sucrose or cane-sugar is very widely distributed in plants, in leaves, stems, roots, fruits, etc It is a white substance which crystallizes well, and is soluble in water and alcohol As previously stated it is hydrolyzed by dilute acids and by invertase into one molecule of glucose and one molecule of laevulose. It is formed by the condensation of glucose and laevulose with the elimination of water. Its constitution is in all probability as follows



so that both the ketone and aldehyde groups are rendered inactive It does not reduce Fehling's solution and does not form an osazone It is dextro-rotatory

Expt 46 *Tests for cane-sugar* The following tests should be made with a 1 % solution of pure crystalline cane-sugar (see also glucose, p 48).

(*a*) *Mooré's test* A negative result is obtained

(*b*) *Fehling's test* No reduction takes place

(*c*) *α-Naphthol test* A positive result is given since sucrose yields laevulose

(*d*) *Hydrolysis* To a few c c of the solution add a drop of strong sulphuric acid and boil for two minutes Then neutralize with caustic soda using litmus as indicator Boil again and add Fehling's solution drop by drop. A reduction takes place owing to the inversion of the cane-sugar by sulphuric acid

(*e*) *Selivanoff's test* A positive result is obtained owing to the liberation of laevulose.

Polysaccharides.

These substances are formed by condensation, with elimination of water, from more than three molecules of monosaccharides

Pentosans

It has already been mentioned that condensation products of the pentoses, the pentosans, are widely distributed The two most frequently occurring pentosans are xylan and araban. No enzymes are known which hydrolyze the pentosans It is characteristic of xylan and araban that they form copper compounds in Fehling's solution in presence of excess of alkali

Araban This pentosan may be regarded as a condensation product of arabinose as already indicated It occurs in various gums (Gum Arabic, Cherry Gum) frequently in combination with other substances On hydrolysis with acids, araban yields arabinose (See also gums and arabinose)

Expt 47 *Preparation of araban from Gum Arabic* (Salkowski, 27) Weigh out 20 gms of gum arabic and dissolve in 500 c c of warm water in a large evaporating dish. Then add 200 c c of Fehling's solution and excess of caustic soda solution The araban will be precipitated as a white gummy mass which will settle at the bottom of the dish. Filter off through muslin Take up the precipitate in dilute hydrochloric acid (1 pt of acid 1 pt of water), and then add alcohol The araban separates out as a white precipitate Wash away the copper chloride with alcohol

Expt 48 *Hydrolysis of araban* The araban from the last experiment is put into a round-bottomed flask with about 200 c.c of 2 % sulphuric acid and heated on a water-bath for 2 hours, the flask being fitted with an air condenser (see p. 45) Then neutralize the liquid with calcium carbonate, filter from calcium sulphate, and concentrate on a water-bath. The sugar is extracted from the syrup with 90 % alcohol Arabinose crystallizes with difficulty but the process may be facilitated by sowing the concentrated alcoholic solution with a few crystals of arabinose. Some of the solution of arabinose should be tested with all the tests given in Expt 38

Xylan. This pentosan occurs in lignified cell-walls, and is the chief constituent of "wood gum." It is found in the wood of many trees (not Coniferae), in bran, in wheat and oat straw, in maize cobs, in the shells of coconuts and walnuts, in the testa of the cotton (*Gossypium*) and in many other tissues also in some gums. On hydrolysis, xylan yields xylose, hence wood shavings, bran, straw, etc , will give the pentose reactions on hydrolysis

Expt 49 *Detection of pentose from pentosans in bran, sawdust and straw* Take a small quantity of bran and boil it up several times with 98 % alcohol, filtering off the alcohol after each treatment This should remove any sugars or glucosides present Allow the alcohol to evaporate off from the bran, and then make the following tests for pentoses (see Expt 38)

(*a*) Heat, for about one minute, a small quantity of the bran in a test-tube, with sufficient concentrated hydrochloric acid to cover it Care should be taken not to char the material Then add as much solid orcinol as will lie on the tip of a penknife Heat gently again for a few seconds Then add one or two drops of strong ferric chloride solution, a green coloration will be produced Add amyl alcohol and the green colour will pass into the alcohol

(*b*) Heat again another portion of the bran with the same quantity of concentrated hydrochloric acid in a test-tube, but this time heat more strongly After heating a few minutes place a piece of filter-paper soaked in a solution of aniline acetate in the mouth of the test-tube A cherry-red coloration will denote the formation of furfural

The above method and tests with bran may be repeated in exactly the same way using sawdust or straw

Expt 50 *Preparation of xylan from sawdust* Extract one kilo of sawdust with 4 litres of 1–2 % ammonia solution for 24 hrs Then filter off the ammoniacal solution through muslin and repeat the extraction The xylan is insoluble in ammoniacal solution, and in this way colouring matters are removed Finally wash the sawdust well with water and press dry from the liquid Then add to the sawdust sufficient 5 % caustic soda solution to make a thick mush (about 1000–1500 c c) and allow it to stand for 24 hrs in a warm place The alkaline solution is then pressed out through calico and filtered through filter-paper To the clear filtrate add an equal volume of 96 % alcohol which will precipitate the xylan as a sodium compound Filter off this precipitate, wash with alcohol, and decompose with alcohol to which a little strong hydrochloric acid has been added to remove the sodium The free xylan is again washed with alcohol, and can be dried by washing with absolute alcohol and ether and finally in a desiccator It is a dirty-white powder which is almost insoluble in water Make the tests for pentoses (see Expt 38) on a little of the solid xylan The reaction will be given in each case.

Expt 51. *Hydrolysis of xylan* Put the xylan obtained in the last experiment in a round-bottomed flask fitted with an air condenser (see p 45) Add 100 c c of 4 % sulphuric acid and heat on a water-bath for 4 hrs Neutralize the solution with calcium carbonate, filter from calcium sulphate and concentrate on a water-bath Test a portion for pentoses (see Expt. 38) and a positive reaction will be obtained To a small quantity add also a few drops of Fehling's solution and boil Reduction will take place

To the remainder of the xylose solution add bromine (see p 45) gradually until there is excess Then remove the excess of bromine by warming on a water-bath Neutralize the solution, which contains xylonic acid, with cadmium carbonate and evaporate on a water-bath Extract the residue with alcohol and filter On concentrating the alcoholic extract, white prismatic needles of cadmium xylonate separate out

It has been shown that pentosans, xylan and probably araban, occur in leaves (Davis, Daish and Sawyer, 15) It is likely that the xylan is widely distributed in all tissues since it forms a constituent of lignified cell-walls

Expt 52 *Detection of pentoses from pentosans in leaves* (Davis, Daish and Sawyer, 15) Take two large leaves of the Sunflower (*Helianthus annuus*) Tear into small pieces and drop into boiling 98 % alcohol in a flask Boil well and filter off the alcohol Repeat until all the green colour is removed Then dry off the alcohol and grind up the leaf residue Perform the test for pentoses (Expt 38 *a* and *c*) on the dry leaf tissue It should give the above tests showing the presence of pentosans

Leaves of the Violet (*Viola odorata*) and Nasturtium (*Tropaeolum majus*) may also be used

Expt 53 *Method for determination of pentosans in tissues, bran and leaves, etc* Weigh out 2 gms of bran, put it into a round-bottomed flask, add 100 c c of 12 % hydrochloric acid and fit the flask with a water condenser Heat gently over wire gauze and distil into a solution of phloroglucin in 12 % hydrochloric acid A green precipitate of furfural phloroglucide is formed which eventually becomes almost black For accurate estimations of pentosans this is filtered off and weighed on a Gooch crucible The same method may be used with leaf residue as in Expt 52

Starches.

Starch. This is a very widely distributed substance in plants. It occurs as solid grains throughout the tissues, in leaves, stems, roots, fruits and seeds It is absent, however, from a number of Monocotyledons, e g *Iris*, Snowdrop (*Galanthus*), *Hyacinthus*, etc It forms one of the chief reserve materials of plants, that is, it is synthesized from sugar when carbon assimilation and carbohydrate synthesis are in progress, and is stored in the solid form in tissues as grains In other circumstances of the plant's existence, when material for metabolism is not available from carbon assimilation, as for instance in germinating seeds or growing bulbs or rhizomes, the starch is hydrolyzed into dextrin and soluble sugar, which is translocated and used as a basis for metabolism. During the night in leaves there is also a similar hydrolysis of the starch which has been temporarily stored from the excess of sugar synthesized during the day.

Starch has a very large molecule and thus a high molecular weight It is insoluble in cold water When heated with a little water it gives starch paste, but on boiling with water it gives an opalescent "solution" which really contains starch in the colloidal state as an emulsoid. In this condition it does not diffuse through dialyzing membranes and does not depress the freezing point of water. The "solution" cannot, strictly

speaking, be filtered, but generally, when hot, it passes to some extent through ordinary filter-paper. Starch is insoluble in alcohol and is precipitated by it

The most characteristic reaction of starch is the blue colour it gives with iodine solution This blue colour disappears on heating, but reappears again on cooling Starch is precipitated from "solution" by half saturation with ammonium sulphate · it does not reduce Fehling's solution

By boiling with dilute acids, starch is first converted into "soluble starch" which still gives a blue colour with iodine. On further boiling, various dextrins (see dextrins) are obtained which give either purple, red or no colour with iodine The final product, after prolonged boiling with acids, is glucose. Hydrolysis with diastase yields dextrin and maltose (see diastase, p 73).

Expt 54 *Preparation of starch from Wheat* Starch may be prepared from a cereal by the following method

Take 25 gms of flour and make it up into a dough with a little water. Allow it to stand for half an hour Then tie a piece of muslin over the top of a beaker which is filled with water Place the dough on the top of the muslin and rub it gently with a glass rod The starch will be separated from the gluten, and will be washed through the muslin and on standing will sink to the bottom of the beaker Allow this to stand till the starch has settled, then decant off the bulk of the liquid Filter off the starch, and wash well with water, then with alcohol and finally with ether Dry in the steam-oven

For the detection of starch in green leaves, see Expt. 77

Expt 55 *Tests for starch* Take a small quantity of the starch prepared in the previous experiment and shake up with a little cold water in a test-tube Filter, and test the filtrate with a drop of iodine (in potassium iodide) solution. No blue colour is obtained Pour a drop of the iodine solution on the residue in the filter It turns deep blue

Weigh out 2 gms of the starch prepared in the last experiment, and mix it into a thin cream with a little water Boil rather more than 100 c.c of water in an evaporating dish, and then gradually add to it the starch paste, keeping the water boiling all the time An opalescent "solution" is obtained With a few c.c of the solution in each case make the following tests

(*a*) Add 1–2 drops of iodine solution A blue colour is obtained Heat the solution the blue colour disappears, but reappears on cooling

(*b*) Add an equal volume of alcohol . the starch is precipitated

(*c*) Add an equal volume of saturated ammonium sulphate solution the starch is precipitated, i e by half saturation with this salt

(*d*) Add basic lead acetate solution . the starch is precipitated

Expt 56 *Hydrolysis of starch* To 50 c c of the starch solution prepared in the last experiment add 1 c c of strong sulphuric acid Boil for 10–20 minutes in a

round-bottomed flask Test a portion of the solution with iodine from time to time ; a purple, red or brown colour is formed due to the dextrin produced in hydrolysis To the remainder of the solution after neutralization, using litmus as indicator, add some Fehling's solution and boil Reduction takes place owing to the glucose formed in hydrolysis

Dextrins

These compounds occur in the plant as transitory substances, since they are formed as intermediate products of the hydrolysis of starch by diastase. They are also formed on heating starch or by boiling it with mineral acids (see previous experiment) The hydrolysis of starch to dextrins is fairly rapid, but the conversion of dextrins into maltose is a much slower process

Both starch and dextrins have the same empirical formula. Various forms of the latter have been identified, such as amylodextrin which gives a blue colour with iodine, erythrodextrin which gives a brownish-red colour with iodine, and achroodextrin which gives no colour with iodine. The dextrins are readily soluble in water, they are precipitated by alcohol but not by basic lead acetate On hydrolysis with acids, they are converted into glucose

Expt 57 *Preparation of dextrin by hydrolysis of starch.* (*a*) *By diastase from leaves of the Pea* (Pisum sativum). Weigh out 10 gms of commercial potato starch and make it into a solution in 250 c c of boiling distilled water as in Expt 55 and cool Then weigh out 10–15 gms. of fresh leaflets of the Pea (*Pisum sativum*) and pound them well in a mortar. Add to the pounded mass 100 c c of distilled water and a few drops of chloroform (see maltase, p 75) and filter The filtrate will contain diastase (see also Expts 78–80) Then add the diastase extract to the starch solution in a flask, plug with cotton-wool and put in an incubator for 48 hrs. If a little of the liquid is withdrawn from time to time and tested with iodine, it will be found that the blue colour due to starch gradually disappears and is replaced by the brownish-red colour due to dextrin After 48 hrs there will be no trace of blue colour, then filter the liquid and concentrate the filtrate on a water-bath to a syrup. Treat the residue with 96–98 % alcohol and filter. A sticky mass of dextrin is left which should be extracted with a little hot alcohol and then reserved for the next experiment To show the presence of maltose, the alcoholic extract is evaporated to dryness on a water-bath, the residue taken up in a little water and the osazone test made (see p. 49) with the solution Crystals of maltosazone will separate out.

(*b*) *By diastase from germinating Barley* (Hordeum vulgare). Grind well 25 gms. of barley grains in a coffee-mill Put the flour into a flask and extract with 96–98 % alcohol by heating on a water-bath This will largely free the grain from sugars. Make a starch "solution" of the residue by boiling with 500 c c. of water and filtering through fine muslin.

Weigh out another 25 gms of barley grains and allow them to germinate by soaking and spreading on damp blotting-paper for 5–7 days Pound the grains well in a mortar, add 100 c c of water, allow to stand for 2–3 hrs and filter Precipitate the filtrate with alcohol and allow to stand for 24 hrs Filter off the precipitate, take up in water and add it to the barley starch "solution," together with a few drops of chloroform Proceed as with (*a*) only the time for hydrolysis may be much shorter, i e 6–12 hrs

Expt 58 *Tests for dextrin* Make a solution of the dextrin prepared in the last experiment and note that it is very soluble in water With the solution make the following tests

(*a*) Add a little iodine solution A reddish-brown colour is produced Heat the solution and the colour will disappear Cool again and the colour will reappear

(*b*) Add an equal volume of strong alcohol The dextrin is precipitated

(*c*) Add an equal volume of saturated ammonium sulphate solution, i e half saturation with ammonium sulphate The dextrin is not precipitated

(*d*) Add some basic lead acetate solution the dextrin is not precipitated

Inulin

Inulin This substance occurs as a soluble "reserve material" in the cell-sap of the underground stems, roots and also leaves of a number of plants, especially members of the Compositae, e g Dahlia (*Dahlia variabilis*), Jerusalem Artichoke (*Helianthus tuberosus*), Chicory (*Cichorium Intybus*) and the Dandelion (*Taraxacum officinale*) It is said to occur also in the Campanulaceae, Lobeliaceae, Goodeniaceae, Violaceae and many Monocotyledons (*Hyacinthus, Iris, Muscari* and *Scilla*)

Inulin is a condensation product of laevulose to which it bears much the same relation as starch to glucose. It is a white substance, soluble in water and insoluble in alcohol It crystallizes out in the cells, in which it occurs, in characteristic sphaero-crystals on addition of alcohol to the tissues. It is hydrolyzed by mineral acids to laevulose. also by the enzyme inulase which occurs in the plant

Expt 59 *Extraction of inulin* Cut off the tubers from two Dahlia (*Dahlia variabilis*) plants, wash well, and put them through a mincing machine Carefully collect the liquid and the crushed tuber, and boil well with sufficient water to cover the crushed material Add also some precipitated calcium carbonate to neutralize any free acids present Then filter through fine muslin, and to the filtrate, which should again be made quite hot, add lead acetate solution until a precipitate (of mucilaginous substances, etc) ceases to be formed Care should be taken to avoid the addition of a large excess of lead acetate Filter off the lead precipitate, and saturate the filtrate with sulphuretted hydrogen till all excess lead is removed Filter off the lead sulphide, neutralize the filtrate to phenolphthalein with ammonia, and evaporate to half bulk or less on a water-bath, when the inulin will probably

begin to deposit Then pour into an equal volume of alcohol, and allow to stand for one or two days The crude precipitate of inulin is filtered off, dissolved in a small amount of water, and reprecipitated with alcohol It can be washed with alcohol and ether and dried over sulphuric acid

The Artichoke (*Helianthus tuberosus*) may also be used, about 12 tubers being necessary

Expt 60 *Tests for inulin* Make a solution of some of the inulin prepared in Expt 59 in hot water It will readily dissolve giving a clear solution With the solution make the following tests

(*a*) Make a very dilute solution of iodine and add to it a drop or two of inulin solution . the brown colour will be unaffected

(*b*) Boil some inulin solution with a little Fehling no reduction takes place

If the inulin solution which is being used should reduce Fehling it indicates that sugar is present as impurity If this is the case, then a little of the solid inulin should be washed free from sugar by means of alcohol before proceeding with the following tests

(*c*) To a little inulin solution add some 1 % alcoholic solution of α-naphthol and a few drops of concentrated sulphuric acid and warm A deep violet colour is produced This is due to the formation of furfural from the laevulose produced in hydrolysis (see laevulose, p. 51)

(*d*) To a little inulin solution add about an equal quantity of strong hydrochloric acid and a few crystals of resorcin. A red coloration is formed This reaction (Selwanoff's test) is also due to the presence of laevulose (see laevulose, p. 51).

Expt 61 *Hydrolysis of inulin.* Some inulin is dissolved in very dilute hydrochloric acid (about 0 5 %) and heated on a water-bath for half an hour in a round-bottomed flask provided with an air condenser (see p 45). The solution is then neutralized with sodium carbonate and concentrated on a water-bath With the concentrated solution make the following tests ·

(*a*) Boil with a little Fehling : the solution is rapidly reduced

(*b*) Make the osazone test (see p. 49) Glucosazone crystals will be found to be formed on microscopic examination (Laevulose forms the same osazone as glucose)

(*c*) Make the tests (*c*) and (*d*) of the last experiment A positive result will be given in each case

Mannans

The mannans which have already been mentioned (see p 50) are condensation products of the hexose, mannose. They occur most frequently, either mixed, or in combination, with the condensation products of other hexoses and pentoses (glucose, galactose, fructose and arabinose) as galactomannans, glucomannans, fructomannans, mannocelluloses, etc Such mixtures or compounds of which mannans form a constituent are widely distributed in the seeds of many plants, i e. Palms (including the Date-palm), Asparagus (*Ruscus*), Clover (*Trifolium*), Coffee Bean (*Coffea arabica*), Onion (*Allium Cepa*) and of members of the Leguminosae,

Rubiaceae, Coniferae and Umbelliferae In seeds the mannans may constitute, together with cellulose, the thickened cell-walls of the endosperm and are included in the term "reserve- or hemi-cellulose" though they are not strictly celluloses "Vegetable ivory," which is the endosperm of the Palm, *Phytelephas macrocarpa*, contains considerable quantities of a mannan and is used as a source of mannose Mannans, in addition, form constituents of certain mucilages, as for instance those in Lily bulbs (*Lilium candidum, L bulbiferum, L Martagon* and others) (Parkin, 23) and tubers of various genera of the Orchidaceae they are also found in the roots of the Dandelion (*Taraxacum*), *Helianthus* and Chicory, *Asparagus* and Clover, and in the wood and leaves of various trees

Many of the mannans, unlike true celluloses, are readily hydrolyzed by dilute hydrochloric and sulphuric acids The mannan in the Coffee Bean, however, is hydrolyzed with difficulty

Galactans

These substances bear the same relationship to the hexose, galactose, as the mannans to mannose, that is, they are condensation products of galactose (see p 49) Similarly they frequently occur, together with the condensation products of other sugars, as galactoaraban, galactoxylan, galactomannan, etc As such they form constituents of many gums and mucilages and of the cell-walls of the reserve tissue of seeds, i.e the Coffee Bean (*Coffea arabica*), the Bean (*Faba*), the Lupin (*Lupinus*), the Paeony (*Paeonia*), the Kidney Bean (*Phaseolus*), the Date (*Phoenix*), the Pea (*Pisum*), the Nasturtium (*Tropaeolum*) and many others (Schulze, Steiger and Maxwell, 29)

Gums

These substances occur widely distributed among plants, especially trees Some gums are wholly soluble in water giving sticky colloidal solutions others are only partially soluble They are all insoluble in alcohol In the solid state they are translucent and amorphous

Chemically the gums are varied in nature, they may in general be regarded as consisting of complex acids in combination with condensation products of various sugars, such as araban, xylan, galactan, etc. On hydrolysis they give mixtures of the corresponding sugars, arabinose, xylose, galactose, etc, in varying proportions, though in some cases one sugar preponderates

Some of the best-known gums are the following

Gum Arabic (*arabin*). This substance is obtained from an Acacia (*Acacia Senegal*), a native of the Soudan The gum exudes from the branches Other species of Acacia yield inferior gums. Gum arabic is a mixture of the calcium, magnesium and potassium salts of arabic acid, a weak acid of which the constitution is unknown, in combination with araban and galactan

Gum Tragacanth This is a product from several Tragacanth shrubs which are species of *Astragalus* (Leguminosae), chiefly *A. gummifer* It is obtained by wounding the stem and allowing the gum to exude and harden On hydrolysis it gives a mixture of complex acids and various sugars such as arabinose, galactose and xylose

Cherry Gum (*cerasin*) occurs in the wood of the stems and branches of the Cherry (*Prunus Cerasus*), the Bird Cherry (*P Padus*), the Plum (*P domestica*), the Almond (*P. Amygdalus*) and other trees of the Rosaceae It exudes from fissures of the bark On hydrolysis it yields almost entirely arabinose.

Expt 62 *Reactions of Gum Arabic* Put a little gum arabic into an evaporating dish and add a little water Heat gently and stir The gum will slowly dissolve, giving a thick sticky solution which does not solidify or gel on cooling Make the following tests, using a little of the gum solution in a test-tube each time

(*a*) Add a little alcohol The gum is precipitated.

(*b*) Add a little Fehling's solution and boil No reduction takes place

The three following experiments show the presence of pentosan complexes in the gum (see also Expt 38, p 44)

(*c*) Add a little phloroglucin to the gum and then strong hydrochloric acid. No colour is produced Now heat, and a cherry-red colour appears

(*d*) Heat the gum solution with a little concentrated hydrochloric acid and then add a trace of orcinol Warm again and then add one or two drops of strong ferric chloride solution. A green coloration will be produced

(*e*) Heat the gum solution strongly with hydrochloric acid, and, after heating for a few minutes, place a piece of filter-paper soaked in a solution of aniline acetate in the mouth of the test-tube A cherry-red coloration indicative of furfural will be formed

Expt 63 *Hydrolysis of Gum Arabic.* Weigh out 10 gms of gum arabic Put it into a round-bottomed flask and add 100 c c of water and 4 c.c of strong sulphuric acid Warm gently until the gum goes into solution Then fit the flask with an air condenser (see p. 45) and heat on a water-bath for about 4 hrs Cool the solution, and neutralize with barium carbonate. Filter off the barium sulphate and concentrate the solution on a water-bath Boil a drop or two of the syrup with Fehling's solution and show that reduction takes place (The original gum either does not reduce Fehling at all, or, if so, only slightly) Then add a little nitric acid

(sp gr 1 15, see Expt 43) to the syrup and heat on a water-bath almost to dryness Pour the residue into about 100 c c of water and allow to stand A microcrystalline precipitate of mucic acid is formed showing the presence of galactose (see p 50) as a product of hydrolysis

Mucilages

The characteristic of these substances is that they swell up in water and produce colloidal solutions which are slimy

Mucilages are widely distributed and may occur in any organ of the plant Sometimes they are confined to certain cells, mucilage sacs or canals They are distinguished from the pectic substances by the fact that they do not gelatinize Some of the best known examples of mucilage-containing tissues are those in the root and flower of the Hollyhock (*Althaea rosea*) in succulent plants (*Aloe, Euphorbia*), in bulbs (*Scilla, Allium*) and tubers (*Orchis Morio*) in seeds of Flax or Linseed (*Linum*) and in fruits of Mistletoe (*Viscum album*).

The mucilages vary in composition They appear to be largely, if not wholly, condensation products of various sugars (galactose, mannose, glucose, xylose, arabinose), similar constituents to those of many gums and hemicelluloses On hydrolysis various mixtures of sugars are produced Of the mucilages, that from linseed has been thoroughly investigated It has been found on hydrolysis to give sugars only, e g arabinose, xylose, glucose and galactose. In this respect mucilages differ from gums, since the latter have always some other accompanying substance in addition to sugars

Expt 64 *Preparation and properties of mucilage from Linseed* (*Linum*) (Neville, 21) Take about 60 gms of linseed and let it soak for 24 hrs in 300 c c of water Then separate the slime from the seeds by squeezing through muslin, and add to the liquid about twice its volume of 96–98 % alcohol The mucilage is precipitated as a thick slimy precipitate Filter off the precipitate and wash with alcohol By washing with absolute alcohol and ether and finally drying in a desiccator, the mucilage may be obtained as a powder

Add water to some of the mucilage It swells up and finally gives an opalescent solution Make with it the following tests

(*a*) Add iodine No colour is given

(*b*) Add a little Fehling's solution and boil. No reduction takes place

Expt 65 *Hydrolysis of Linseed mucilage* Put the remainder of the mucilage in a round-bottomed flask and add 50 c c of 4 % sulphuric acid Fit the flask with an air condenser (see p 45) and heat for at least four hours on a water-bath. Cool and neutralize with barium carbonate Filter off the barium sulphate, and

concentrate the filtrate on a water-bath With the concentrated solution make the following tests

(*a*) Add a few drops to a little boiling Fehling solution Reduction immediately takes place

(*b*) Make the phloroglucm, orcinol and furfural tests for pentoses, using a small quantity only of the hydrolysis mixture for the tests A positive result will be given in each case The pentoses, arabinose and xylose, are responsible for these reactions

(*c*) Add to some of the solution phenylhydrazine hydrochloride, sodium acetate and a little acetic acid, and leave in boiling water for half an hour for the osazone test [see Expt 41 (*d*)] A mixture of osazones will separate out, among which glucosazone can be identified

(*d*) Concentrate the remainder of the solution and then add some nitric acid of sp gr 1 15 (see Expt 43) Evaporate down on a water-bath to one-third of the bulk of the liquid and then pour into about 100 c c of water A white microcrystalline precipitate of mucic acid will separate out, either at once or in the course of a day or two This demonstrates the presence of galactose

Pectic substances

These substances are considered at this point since they are said to constitute, in more or less intimate connexion with cellulose, the middle lamella of cell-walls in many tissues The pectic substances are frequently found in the juices of succulent fruits in which the tissues have disintegrated, such as red currants and gooseberries. They have been isolated chiefly from fleshy roots, stems or fruits, as, for instance, from turnips, beetroot, rhubarb stems, apples, cherries and strawberries.

Recent work (Schryver and Haynes, 28) points to the fact that in turnips, strawberries, rhubarb stems and apples, there is the same pectic material, and it is possible that all such substances may be identical. The compound isolated in the above case is of an acidic nature and has been termed pectinogen When pectinogen is treated with dilute solutions of caustic alkali at ordinary temperatures, it is rapidly changed into a second substance termed pectin, which is readily converted into a gel under certain conditions.

In the case of juicy fruits, such as currants and gooseberries, the pectinogen can be precipitated as a gelatinous precipitate by adding alcohol to the expressed juice. In the case of fleshy fruits, stems and roots, the procedure is as follows. The tissues are thoroughly disintegrated in a mincing machine and pressed free from all juice in a powerful press The residue is then dried, finely ground, washed with water and finally extracted with dilute ammonium oxalate solution in which pectinogen is soluble. The extract is concentrated and the

pectinogen precipitated by alcohol It may be purified by reprecipitation

Pectinogen is precipitated from aqueous solution by alcohol as a very bulky gelatinous mass, but when dried it forms an almost colourless granular powder Put into water it absorbs large quantities of liquid and dissolves slowly, giving an opalescent solution with a distinctly acid reaction

As mentioned above pectinogen in alkaline solution is rapidly converted into pectin. A solution of pectinogen is not precipitated either by acid or dilute solutions of calcium salts but, after treatment with alkali and conversion into pectin, both the aforesaid reagents produce gelatinous precipitates. A similar precipitate is also formed when lime water is added in excess to a solution of pectinogen and it is allowed to stand There is little doubt that the pectinogen is converted by the alkali into pectin Pectin is also an acid substance and it is insoluble in water, giving an insoluble salt with calcium After treatment of pectinogen with alkali the pectin can, as already stated, be precipitated by adding acid.

Analyses of pectin from various sources have led to the suggestion of $C_{17}H_{24}O_{16}$ as its formula There is also evidence that it contains one pentose group This can be detected and estimated by the furfural phloroglucide method (see Expt 53)

Expt 66 *Extraction and reactions of pectinogen* Take about half a pound of red currants and squeeze out the juice through fine muslin into a large beaker Then add to the juice about 2–3 times its bulk of 96–98 % alcohol A bulky gelatinous precipitate of pectinogen will separate out Allow the precipitate to stand for a time in the alcohol, and then filter off Wash with alcohol and finally press free from liquid. Dissolve the precipitate in as little water as will enable it to go into solution To two small portions of the solution add respectively (*a*) a few drops of strong hydrochloric acid, (*b*) an excess of calcium chloride solution Note that no precipitate is formed in either case

Expt 67 *Conversion of pectinogen into pectin, and reactions of pectin.* Take about one-third of the pectinogen solution prepared in Expt 66, make it alkaline with caustic soda, and let it stand for about 10–15 minutes Then divide the solution into two parts and add respectively (*a*) sufficient hydrochloric acid to acidify, (*b*) excess of calcium chloride solution. In the first case a gel of pectin is formed in the second case a gelatinous precipitate of the calcium salt of pectin

To a further quantity of the pectinogen add excess of lime water and let it stand The gelatinous calcium precipitate will separate out in a short time.

Expt 68 *Detection of the pentose group in pectinogen* Filter off the pectin gel obtained in the last experiment and allow it to dry Then test for the pentose group by the orcinol, phloroglucinol and furfural tests (see Expt 38). All results will be found to be positive

The extraction of pectinogen, etc. in the above experiments can equally well be carried out with other material, e g ripe gooseberries, raspberries and strawberries, using exactly the same methods.

Expt. 69 *Preparation of pectinogen from Turnips* Take two full-sized turnips and mince them finely in a mincing machine Then wrap the mass in a piece of strong unbleached calico and press out the juice as completely as possible in a press The juice contains little pectinogen and can be thrown away The pressed mass is then thrown into 0 5 % ammonium oxalate solution heated to 80–90° C. on a water-bath and stirred to make a paste The liquid is again rapidly pressed out in the press To the viscid extract an equal volume of 96 % alcohol is added, and the pectinogen separates out as a voluminous gelatinous precipitate This is filtered off and, when pressed free from alcohol and dried, can be used for tests as in the previous experiments

The gelatinization of pectinogen can also be brought about by certain enzymes termed pectases which are found in the juices of various plants, i.e root of Carrot (*Daucus Carota*) and leaves of Lucerne (*Medicago sativa*), Lilac (*Syringa vulgaris*) and Clover (*Trifolium pratense*)

Expt 70 *Action of pectase on pectinogen* Make an extract of either Lucerne or Clover leaves by pounding them in a mortar with a little water, and then filter Add the filtrate to some of the pectinogen solution prepared in Expt 66 or 69. On standing a gelatinous precipitate will be produced Should the reaction be slow, it may be accelerated by placing the mixture in an incubator

Celluloses.

Celluloses are very important polysaccharides They form constituents of the structural part of all the higher plants The cell-wall of the young cell consists entirely of cellulose, but in older cells the walls may be lignified, cuticularized, etc., i.e. the cellulose may be accompanied by other substances such as lignin, cutin, mucilage, etc. In the light of these facts the term cellulose is made to include

1. Normal celluloses
2. Compound celluloses.
   - (*a*) Ligno-celluloses
   - (*b*) Pecto-celluloses.
   - (*c*) Adipo- or cuto-celluloses.
3. Pseudo- or Reserve celluloses.

True or normal cellulose. Of this substance, as we have said, many cell-walls are composed The most familiar form of cellulose is cotton, which consists of hairs, each being a very long empty cell, from the testa or coat of the seed of the Cotton plant (*Gossypium herbaceum*).

Crude cotton (i e the hair cell-walls) is not quite pure cellulose, but contains a small amount of impurity from which it is freed by treatment first with alkali and subsequently with bromine or chlorine. All kinds of cotton material, cotton-wool, and the better forms of paper (including filter-paper) may be regarded as almost pure cellulose

Pure cellulose is a white, somewhat hygroscopic, substance. It is insoluble in water and all the usual solvents for organic substances It is, however, soluble in a solution of zinc chloride in hydrochloric acid in the cold, and in a solution of zinc chloride alone on warming It is also soluble in ammoniacal cupric oxide (Schweizer's reagent).

In addition cellulose is soluble in concentrated sulphuric acid, which on standing converts it first into a hydrate and then finally into glucose If, however, water is added to the sulphuric acid solution as soon as it is made, the gelatinous hydrate of cellulose is precipitated. This substance is termed "amyloid" since it gives a blue colour with iodine Concentrated nitric acid converts cellulose into nitrates, of which one is the substance, gun-cotton In 10 % alkalis cotton fibres thicken and become more cylindrical This procedure has been employed by Mercer to give a silky gloss to cotton, and the resultant product is called mercerized cotton

Expt 71 *The colour tests and solubilities of cellulose*

(*a*) Dip a little cotton-wool into a solution of iodine in potassium iodide Then put the stained wool into an evaporating dish and add a drop or two of concentrated sulphuric acid A blue coloration is given This is due to the formation of the hydrate "amyloid" mentioned above

(*b*) Dip some cotton-wool into a calcium chloride iodine solution (To 10 c c. of a saturated solution of calcium chloride add 0 5 gm of potassium iodide and 0 1 gm. of iodine Warm gently and filter through glass-wool) A rose-red coloration is produced which eventually turns violet

(*c*) Heat a strong solution of zinc chloride (6 pts of zinc chloride to 10 pts of water) in an evaporating dish and add 1 part of cotton-wool The cellulose will in time become gelatinized, and if a little water is added from time to time, a solution will eventually be obtained on continuous heating

(*d*) Make a solution of zinc chloride in twice its weight of concentrated hydrochloric acid and add some cotton-wool The wool will rapidly go into solution in the cold

(*e*) Add some cotton-wool to an ammoniacal copper oxide solution and note that it dissolves (To a strong solution of copper sulphate add some ammonium chloride and then excess of caustic soda Filter off the blue precipitate of cupric hydroxide, wash well, dry thoroughly, and dissolve in strong ammonia) Add strong hydrochloric acid and the cellulose is precipitated out again Then add water and wash the precipitate until it is colourless Test the roughly dried precipitate with a little iodine and strong sulphuric acid A blue coloration is given

All the above tests may be repeated with threads from white cotton material, with filter-paper and good white writing paper

Try tests (*a*) and (*b*) with newspaper, and note that they are not so distinct as with writing paper owing to the presence of ligno-cellulose (see Expt 73)

Expt 72 *Hydrolysis of cellulose by acid* Dissolve as much filter-paper as possible in 5 c c of concentrated sulphuric acid and when all is in solution pour into 100 c c of distilled water Boil the solution in a round-bottomed flask fitted with an air condenser (see p 45) and use a sand-bath for heating. After boiling for an hour, cool and neutralize the solution with solid calcium carbonate Add a little water if necessary and filter Test the filtrate with the following tests

(*a*) Make the osazone [see Expt 41 (*d*)] Note that crystals of glucosazone are formed

(*b*) Add a little Fehling's solution and boil Note that reduction takes place

Instead of using filter-paper, the above experiment may also be carried out with cotton-wool or threads from white cotton material.

Ligno-cellulose. As the cells in plants grow older the walls usually become lignified, that is part of the cellulose becomes converted into ligno-cellulose. The extreme amount of change is found in wood The least amount in such fibres as those from the stem of the Flax (*Linum usitatissimum*) which, when freed from such impurities, consist of cellulose only and constitute linen. Other fibres, containing more ligno-cellulose, are those of the stem of the Hemp plant (*Cannabis sativa*) and the Jute plant (*Corchorus*) from which string, rope, canvas, sacking and certain carpets are made The percentages of pure cellulose in these various lignified tissues are as follows

Cotton fibre	88 3 %
Flax and Hemp fibre ...	72–73 %
Jute	54 %
Beech and Oak wood . .	35–38 %

The ligno-celluloses are generally regarded as consisting of cellulose and two other constituents, of which one contains an aromatic nucleus and the other is of the nature of a pentosan (see xylan, p. 53) Both are sometimes classed together and termed lignin or lignon The lignin reactions (see below) depend on the presence of an aromatic complex. It has been suggested that coniferin, vanillin and allied compounds which are present in wood are probably the substances responsible for the reaction (Czapek, 6).

Although the best paper is made from cellulose, cheaper forms of paper are manufactured from ligno-cellulose, and, as a result, they give reactions for lignin and are also turned yellow by exposure to light.

Expt 73 *Reactions of lignin.*

One of the most striking reactions of lignin (due as it is supposed to a furfural

grouping) is the magenta-red coloration given by phloroglucin in the presence of concentrated hydrochloric acid

Soak the tissue to be experimented upon with an alcoholic solution of phloroglucin and then add a drop or two of strong hydrochloric acid The magenta-red colour will be produced

As material, practically any lignified tissue may be used Shavings from twigs of any tree or shrub, e g pith and wood from the Elder (*Sambucus nigra*), will be found useful also shavings from a match, straw, bran, coarse string, cheap white paper, such as newspaper or white and pale-coloured papers used for wrappings

Make the phloroglucin test on good white writing paper It should not give the reaction since it is made from cellulose

Other phenols and their derivatives will also give colour reactions with lignin in the presence of hydrochloric acid, but the colorations in most cases are not so much developed as with phloroglucin (Czapek, 6) For this reason (though it is also possible to use any of the lignified tissues suggested above) good results are obtained by using strips of any cheap newspaper, since the reagents seem to penetrate this material quickly

Soak strips of newspaper (or other material) in alcoholic solution of the following substances, or such of them as are available, and then add a few drops of concentrated hydrochloric acid It is useful to put the material on a white glazed tile or plate

	Reaction
Phenol	blue-green coloration
Resorcinol	violet ,,
Orcinol	red-violet ,,
Catechol	greenish-blue ,,
Pyrogallol	blue-green ,,
Guaiacol	yellow-green ,,
Cresol	greenish ,,
α-Naphthol	greenish ,,
Thymol	green ,,
Indol	cherry-red ,,
Skatol	cherry-red ,,

It should be noted that strong hydrochloric acid alone will sometimes give a red colour with woody tissues this is due to the presence of phloroglucin in the wood itself (see phloroglucin, p. 88)

Expt 74 *Destruction of the lignin element in wood* Take some paper which gives the phloroglucin reaction for lignin strongly and cut it up into pieces about an inch square Then boil the paper in some 1 % sodium hydroxide solution for a short time After washing well, put it into a flask or large test-tube with a few c.c of bromine-water and allow it to stand for an hour or two Then wash again and heat in a 2 % solution of sodium sulphite Wash free from sulphite, dry and test with alcoholic phloroglucin solution and strong hydrochloric acid No red colour, or very little, will be produced If a little red coloration is formed, the process should be repeated until finally all the lignin reaction disappears

Pecto-cellulose. The non-cellulose constituents in this case belong to the class of pectic substances which have already been considered (see p. 63). Such celluloses occur in the cell-walls of the tissues of many fleshy roots, stems and fruits.

Adipo- and cuto-celluloses. These products are found in the walls of corky and cuticularized tissues Their chemical composition is obscure but they appear to contain substances of a fatty or wax-like nature

Hemi-celluloses. These are not strictly celluloses since they are built up of mannans, galactans and pentosans on lines which have already been considered (see pp 59 and 60). They frequently occur united with each other, for instance as galacto-, gluco- and fructomannan, galactoaraban, galactoxylan, etc They are found in the cell-walls of the tissues of many seeds.

The Synthesis and Inter-relationships of Carbohydrates in the Plant.

Now that the properties and characteristics of various carbohydrates have been dealt with, their synthesis and their relationships, one to another, may be considered

In the previous chapter it has been shown how the plant synthesizes a sugar from carbon dioxide and water by virtue of the chemical energy obtained from transformation of radiant energy by means of chlorophyll. When this sugar reaches a certain concentration in the cell, in the majority of plants, starch is synthesized from it by condensation with elimination of water. The starch is thus the first visible product of assimilation and is temporarily "stored" in an insoluble form during the day, when photosynthesis is active. During the night photosynthesis ceases but the sugar is still translocated from the leaf, as it was in fact during the day, thus, since the supply ceases, the concentration in the cell falls, and the "stored" starch is then hydrolyzed again into sugar, and the process continues until the leaf is either starch-free, or contains considerably less starch. During the next day, the starch formation is repeated and so forth The process of hydrolysis of starch is carried out by the enzyme, diastase, with the formation of dextrin and maltose. In all probability this same enzyme controls the synthesis of starch.

On the other hand, it has been shown that many plants do not form starch at all in their leaves but only sugar. Examples are the adult

Mangold plant (*Beta vulgaris*) and many Monocotyledons (*Allium, Scilla*).

As to the question of which sugars are present in the leaf, there is only evidence from accurate work on a few plants Careful investigations have been made of the sugars in leaves of the Mangold (*Beta vulgaris*) (Davis, Daish and Sawyer, 15), Garden Nasturtium (*Tropaeolum majus*) (Brown and Morris, 5), the Snowdrop (*Galanthus nivalis*) (Parkin, 24), the Potato (*Solanum tuberosum*) (Davis and Sawyer, 17) and the Vine (*Vitis vinifera*) The general conclusions drawn from these investigations are that sucrose, glucose, and laevulose are always present in leaves that maltose results from the hydrolysis of starch, being absent from leaves which do not form starch Maltose is not present in appreciable quantity even in starch-producing leaves because it is rapidly hydrolyzed into glucose by maltase (In such cases where it has been detected it has been due to diastase action during the drying of leaves before extraction) Other leaf carbohydrates are the pentoses which have been found in a good many species examined and may be widely distributed, the pentosans, their condensation products, also occur as well as dextrin (Potato)

The next question to be considered is what sugar is first synthesized in the leaf. Is it glucose, laevulose, sucrose or maltose? It is known that the enzymes, invertase and maltase, are commonly present in leaves and that these enzymes respectively control the hydrolysis, of cane-sugar into glucose and laevulose, and of maltose into glucose. It is also possible that they respectively control the synthesis of sucrose and maltose Laevulose, likewise, as may be supposed, can be obtained from glucose Thus all the sugars can be readily converted one into another, but to ascertain which is the first product of synthesis is not an easy problem

In addition to the above-mentioned work on the nature of the sugars present in leaves, a good deal of careful analysis has been made as to the proportions in which the sugars occur relatively to each other during stated periods of time, with a view to answering the question as to which is the first-formed sugar There are two possibilities: one, that it is sucrose and that it is hydrolyzed into glucose and fructose the other, that it is glucose, from which fructose is derived, and the two are then synthesized to form sucrose

Opinion is divided on this point and there is not at present sufficient experimental evidence to decide the matter The majority of investigators regard sucrose as the first-formed sugar, and suggest that it is inverted into hexoses for purposes of translocation, since the smaller

molecules would diffuse faster There is experimental evidence that there is an increase in hexoses in the conducting tissues Others favour the view that glucose is the first-formed sugar, and bring forward evidence to this effect There is however no reason why hexoses should not be formed first and then converted into cane-sugar and temporarily stored as such, being again reinverted into hexoses for translocation. Nor is there any reason for supposing that the first-formed sugar is always the same in every plant

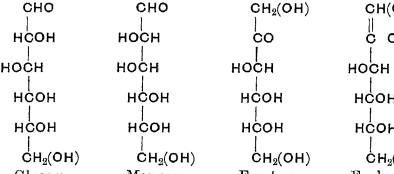
There appears to be very little doubt that maltose is formed in the hydrolysis of starch, and also that starch is a temporary reserve material in the leaves, but whether formed direct from sucrose or from hexoses cannot be stated

There is some evidence in favour of the view that glucose is more readily used in respiration than laevulose, for under circumstances when neither can be increased, the glucose tends to disappear

From the leaf the various sugars are translocated to other organs of the plant, e g root, stem, flower, fruit and seed In some cases starch is synthesized from the sugars and "stored" in roots, tubers, tuberous stems, fruits and seeds In other cases the sugars themselves may be "stored," as, for instance, in the root of the Beet (*Beta vulgaris*), or they may have a biological significance, as in sweet fruits. It must also be borne in mind that sugars are employed throughout the plant in respiration and in the synthesis of more complex substances, i.e cellulose, gums, pentosans, mucilage, aromatic substances, fats and to a certain extent proteins. in fact they or their precursors constitute the basis from which all organic compounds are synthesized

The following experiments can be performed with either the Garden Beet or the Mangold Wurzel, both of which are varieties of *Beta vulgaris*, the Common Beetroot The sugars in the leaves and petioles of the Mangold have been investigated (Davis, Daish and Sawyer, 15) and sucrose, laevulose and glucose have been found. Starch is absent in the adult plant and also maltose. The opinion is held that sucrose is the first-formed sugar of photosynthesis and that this is hydrolyzed for translocation on account of the greater rate of diffusion of the smaller molecules of glucose and laevulose. These are again synthesized in the root to form sucrose where the latter is stored, and hexoses are almost absent from this organ Though the facts concerning the distribution of the sugars stated above are reliable, it is not certain that the deductions are permissible. The leaf contains the enzymes, invertase, maltase and diastase (Robertson, Irvine and Dobson, 25)

In connexion with the occurrence of various sugars in leaves it is of interest to note that glucose, fructose and mannose can pass over into one another in alkaline aqueous solution This has been explained by their conversion into the enolic (unsaturated) form common to all three hexoses



Expt. 75 *To show the presence of both hexoses and sucrose in the leaf* (Davis, Daish and Sawyer, 15) Take about 5 gms of fresh leaf of either the Beet or Mangold (Leaves of the Garden Nasturtium (*Tropaeolum majus*) and Wild Chervil (*Chaerophyllum sylvestre*) may also be used) Tear them into small pieces and drop them into boiling 90–98 % alcohol in a flask on a water-bath. In this way the enzymes of the leaf are killed, and no changes will occur in the carbohydrates present After boiling for a short time, the alcohol is filtered off and the extraction repeated. Evaporate the filtrate to dryness in an evaporating dish on a water-bath The filtrate will contain chlorophyll and various pigments, sugars, glucosides, aromatic compounds and other substances according to the plant used. Then add about 20 c c of water and at intervals a few drops of basic lead acetate until it ceases to form a precipitate By this means all hexoses *combined* with aromatic substances as glucosides (see p 142) are precipitated as insoluble lead salts The precipitate is filtered off and the lead in the filtrate removed by sodium carbonate, avoiding excess Filter again and the filtrate will contain the sugars Boil the latter and add Fehling's solution drop by drop till reduction ceases Filter off the copper oxide and then boil the solution with dilute sulphuric acid for a few minutes and make neutral to phenolphthalein Reduction will occur on adding more Fehling and boiling, owing to the inversion of the cane-sugar present

Expt. 76 *To show the presence of hexoses in the leaf by means of the formation of glucosazone* Leaves of *Beta*, *Chaerophyllum sylvestre*, or *Tropaeolum* may be used Extract as in the previous experiment and precipitate the glucosides with the minimal amount of basic lead acetate. Test for osazone in the filtrate as in Expt 41 (*d*).

Expt 77 *To obtain starch from green leaves* Weigh out 25 gms of leaflets of the Pea (*Pisum sativum*) The leaves should have been picked in the evening after a sunny day, and it does not matter if the cut leaves are left overnight Dip the leaflets for a moment into boiling water, remove excess of water and drop them into 200 c c of 96–98 % alcohol and boil till the chlorophyll is extracted . then filter.

Take the residue of leaves and pound (but not finely) in a mortar and then wash thoroughly with distilled water Filter through muslin and press free from water (this process extracts most of the protein) Boil the residue with 100 c c of water and filter To the filtrate add iodine At first the colour may disappear owing to the presence of protein in solution in addition to the starch When more iodine is added a deep blue coloration is formed

Plant Enzymes which hydrolyze Carbohydrates

Diastase. In the plant starch may be regarded as a reserve product It is synthesized from sugar, and may be again hydrolyzed into sugar. It can be shown experimentally that starch is converted into glucose by boiling with acids, but in the plant the hydrolysis of starch is catalyzed by the enzyme, diastase Although the reaction is doubtless of considerable complexity, it may, broadly speaking, be represented as follows

$$(C_6H_{10}O_5)_n + H_2O \longrightarrow \underset{\text{Dextrin}}{(C_6H_{10}O_5)_x} + \underset{\text{Maltose}}{C_{12}H_{22}O_{11}}$$

Thus the final products under these conditions are dextrin and the disaccharide, maltose, and not glucose.

It is reasonable to assume that cells which contain starch also either contain, or are capable of producing, diastase. But the amount of diastase present, or at any rate capable of being extracted, varies in different tissues Diastase, like most enzymes, is soluble in water. In many cases, however, a water-extract from fresh crushed tissues in which diastase occurs, will not contain any appreciable amount of enzyme. This is sometimes due to the fact that the protoplasm does not readily yield up the enzyme until it has been killed If the tissues are dried at a moderate temperature (30–40° C) both the powdered leaves themselves and a water extract are fairly rich in diastase, or, if the living tissues are macerated and extracted with water to which chloroform has been added, the cells die more rapidly and yield up the enzyme to the solvent From such a water extract, a crude precipitate containing the enzyme may be obtained by addition of alcohol For obtaining the maximum results with diastatic activity in leaves, a water extract should be made after they have been killed, either by drying, or by the action of toluol or chloroform.

It has been shown (Brown and Morris, 5) that in leaves which contain tannin, the presence of the latter largely inhibits the action of the enzyme and may be the cause, in such cases, of an entire lack of activity in the extract.

The diastatic activity of leaves appears to vary largely in different genera and species The subject has been investigated (Brown and Morris, 5) and a list of their relative activities has been drawn up as follows

[The numbers represent the amount of maltose, expressed in grams, which 10 gms of air-dried leaf will produce from soluble-starch (starch treated with dilute hydrochloric acid) by hydrolysis in 48 hrs at 30° C]

Pisum sativum	240 30	Helianthus annuus .	3·94
Phaseolus multiflorus	110 49	H tuberosus .	3 78
Lathyrus odoratus . .	100·37	Funkia sinensis	5 91
L pratensis .	34·79	Allium Cepa .	3 76
Trifolium pratense	89 66	Hemerocallis fulva.	. 2 07
T ochroleucum	56 21	Populus sp . .	3·79
Vicia sativa .	79 55	Syringa vulgaris . . .	2·53
V hirsuta ..	53 23	Cotyledon Umbilicus	4 61
Lotus corniculatus	. 19 48	Humulus Lupulus	2 01–9 60
Lupinus sp. .	. 3 51	Hymenophyllum demissum	4 20
Grass with Clover	27 92	Hydrocharis Morsus-ranae .	0 267
Tropaeolum majus .	3 68–9 64		

From the above table it is seen that the leaves of genera of the Leguminosae are apparently very rich in diastase Whether this is so, or whether in other plants the diastatic activity is inhibited by other substances, has not yet been ascertained As mentioned above, tannins inhibit the action of diastase, and hence leaves rich in tannin, e.g. Hop (*Humulus*), cannot be expected to yield good results.

The tissues of germinating barley (*Hordeum vulgare*) also contain large quantities of diastase, and this material can be used to demonstrate the solubility, isolation and activity of the enzyme.

The action on starch of diastase from the leaf of the Common Pea (*Pisum sativum*) and from germinating barley grains has already been demonstrated [see Expt. 57 (*a*) and (*b*)] in connexion with dextrin The following experiments have special reference to the enzyme.

Expt 78 *To demonstrate the activity of diastase from germinating barley.* Grind 2–3 gms of barley grains in a coffee-mill Boil the product with 100 c.c. of water and filter, first through fine muslin if necessary, then through filter-paper. A starch "solution" will be obtained

Pound up 2–3 gms of germinated barley grains in a mortar and extract the mass with 50 c c of water Filter, and take two equal portions in two test-tubes. Boil one tube To both tubes add an equal quantity of the starch solution prepared above. Place the tubes in a beaker of water at 38–40° C From time to time withdraw a drop from each tube with a pipette and test with iodine solution on a white tile. The starch in the unboiled tube will gradually give the dextrin reactions (see p. 57); that in the boiled tube will remain unchanged

This simple method may also be adopted for showing the diastatic activity of leaves Instead of germinating barley, a few leaflets of the Pea (*Pisum sativum*) or Clover (*Trifolium pratense*) should be pounded up in a mortar and extracted with 50 c c of water and filtered

Expt 79 *To show that leaf-diastase is still active after drying the leaves at temperatures not higher than* 38° C Take 10 gms of fresh Pea leaves and dry by spreading them in the sun Then powder and finally dry in an incubator at 38° C Make up 500 c c of a 1 % solution of starch (see Expt 55) To this add the dry leaf powder together with a few drops of toluol and keep in an incubator at 38° C Test the solution with iodine from time to time and note the hydrolysis of the starch

Expt 80 *To show that the action of diastase is impaired by contact of the enzyme with alcohol.* Pound up 10 gms of fresh Pea leaves, add 100 c c of water, a few drops of toluol and allow the mixture to stand for 12 hrs Filter off the extract, and add at least an equal bulk of 96–98 % alcohol A white precipitate is produced which, among other substances, contains crude diastase Filter, and wash the precipitate with a little water into 500 c.c of a 1 % starch solution Add a few drops of toluol, plug with cotton-wool and put in an incubator Test with iodine from time to time It will be found that the hydrolysis takes place much more slowly than in the previous experiments

Expt 81 *To show that the action of diastase is inhibited by tannic acid* Take about 0 5–1 gm of dried powdered Pea leaf. Let it stand for about 12 hrs in 50 c c of water containing a few drops of toluol Filter off, and to equal amounts of the filtrate in two small flasks add about 10 c.c of a 1 % starch solution Add also 10 c c of a 0 5 % tannic acid solution to one test-tube Put both tubes into an incubator Test with iodine solution after a few hours It will be found that the tannic acid has inhibited the action of the diastase

Maltase. This enzyme hydrolyzes maltose into two molecules of glucose:

$$C_{12}H_{22}O_{11} + H_2O = 2C_6H_{12}O_6$$

Investigations upon maltase have, until recently, produced rather contradictory results, but later work (Davis, 12 Daish, 13, 14) has led to more satisfactory conclusions. The latter show that maltase is most probably present in all plants in which hydrolysis of starch occurs It has been detected in leaves of the Nasturtium (*Tropaeolum*), the Potato (*Solanum*), the Dahlia, the Turnip (*Brassica*), the Sunflower (*Helianthus*) and the Mangold (*Beta*), and it is most probably widely distributed in foliage leaves. Its detection is not easy for various reasons which are as follows. It is not readily extracted from the tissues by water: it is unstable, being easily destroyed by alcohol and chloroform. Its activity is also limited or even destroyed at temperatures above 50° C. Hence the extraction of maltase, by merely pounding up tissues with water, does not yield good results moreover, as an antiseptic, toluol must be

used and not chloroform Finally, if the enzyme is to be extracted from dried material, this must not be heated at too high a temperature previous to the extraction

Maltase occurs in quantity in both germinated and ungerminated seeds of cereals. If, in kilning, malt has not been heated at too high a temperature, the maltase may not be destroyed, and, in such cases, malt extract will contain both diastase and maltase. This would explain the fact that glucose, instead of maltose, has sometimes been obtained by the action of malt diastase on starch. In other cases, when a higher temperature has been employed, the maltase will be destroyed Maltase itself, of course, does not act directly upon starch but only on maltose The use of chloroform, as an antiseptic, by some observers explains how they came to overlook the presence of maltase, thus obtaining maltose, and not glucose, as an end product in hydrolysis by malt extracts The optimum temperature for the maltase reaction is 39° C

The presence of maltase in leaves is not readily shown for the following reasons Since maltase is destroyed by alcohol, the preparation of a crude precipitate of the enzyme by precipitating a water extract of the leaves is not satisfactory. If the water extract is added directly to maltose, and incubated, hydrolysis may be demonstrated by determining the reducing power of the sugars formed A control experiment must, however, be made by incubating the water extract alone, and subsequently determining the reducing power of any sugars present

Invertase. This enzyme hydrolyzes cane-sugar into one molecule of glucose and one molecule of laevulose.

$$C_{12}H_{22}O_{11} + H_2O = C_6H_{12}O_6 + C_6H_{12}O_6$$

Invertase is probably very widely distributed in plants. Its presence has been demonstrated in the leaves and stem, though not in the root, of the Beet (*Beta vulgaris*) (Robertson, Irvine and Dobson, 25). Also in the leaves of a number of other plants (Kastle and Clark, 20). Its detection, by its action on sucrose, is not easy on account of the presence of other enzymes and reducing sugars in leaf extracts.

The absence of invertase from the root of the Beet raises a difficulty as to how the cane-sugar is synthesized from the hexoses supplied from the leaves (see p 71). Some observers (Robertson, Irvine and Dobson, 25) incline to the view that cane-sugar is synthesized in the stems and travels as such to the roots Others (Davis, Daish and Sawyer, 15) maintain that the cane-sugar is synthesized in the root, even though invertase is absent

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CHAPTER VI

THE FATS AND LIPASES

A FAT may be defined as an ester or glyceride of a fatty acid Just as an inorganic salt, such as sodium chloride, is formed by the reaction of hydrochloric acid with sodium hydroxide, so a fat is formed by the reaction of the trihydric alcohol, glycerol, and a fatty acid

The word *fat* is not a familiar one in botanical literature, the term *oil* being more commonly used It is generally met with in connexion with the reserve products of seeds The oils of seeds are, however, true fats The term oil may be misleading to some extent, because a fat which is liquid at ordinary temperatures is usually spoken of as an oil, and yet there are also many other substances, of widely differing chemical composition, which have the physical properties of oils, and which are known as such.

Most of the vegetable fats are liquid at ordinary temperatures but some are solids.

The best-known series of acids from which fats are formed is the series $C_nH_{2n}O_2$ of which formic acid is the first member. The other members of the series which occur in fats are:

Acetic acid	CH_3COOH	or	$C_2H_4O_2$
Butyric acid	C_3H_7COOH	or	$C_4H_8O_2$
Caproic acid	$C_5H_{11}COOH$	or	$C_6H_{12}O_2$
Caprylic acid	$C_7H_{15}COOH$	or	$C_8H_{16}O_2$
Capric acid	$C_9H_{19}COOH$	or	$C_{10}H_{20}O_2$
Lauric acid	$C_{11}H_{23}COOH$	or	$C_{12}H_{24}O_2$
Myristic acid	$C_{13}H_{27}COOH$	or	$C_{14}H_{28}O_2$
Palmitic acid	$C_{15}H_{31}COOH$	or	$C_{16}H_{32}O_2$
Stearic acid	$C_{17}H_{35}COOH$	or	$C_{18}H_{36}O_2$
Arachidic acid	$C_{19}H_{39}COOH$	or	$C_{20}H_{40}O_2$
Behenic acid	$C_{21}H_{43}COOH$	or	$C_{22}H_{44}O_2$

Another series is the oleic or acrylic series $C_nH_{2n-2}O_2$ of which the members are

Tiglic acid	$C_5H_8O_2$
Oleic acid	$C_{18}H_{34}O_2$
Elaidic acid	$C_{18}H_{34}O_2$
Iso-oleic acid	$C_{18}H_{34}O_2$
Erucic acid	$C_{22}H_{42}O_2$
Brassidic acid	$C_{22}H_{42}O_2$

Of these, oleic acid (as glyceride) is the most widely distributed. Yet other series are

The linolic	$C_nH_{2n-4}O_2$
The linolenic	$C_nH_{2n-6}O_2$
The clupanodonic	$C_nH_{2n-8}O_2$
The ricinoleic	$C_nH_{2n-2}O_3$

The fat which occurs in an oil-containing seed is not composed of the glyceride of one acid, but is a mixture of the glycerides of several, or even a large number of different acids, often members from more than one of the above series Thus the fat of the fruit of the Coconut (*Cocos nucifera*) consists of a mixture of the glycerides of caproic, caprylic, capric, lauric, myristic, palmitic and oleic acids. Linseed oil from the seeds of *Linum usitatissimum* again is a mixture of the glycerides of palmitic, myristic, oleic, linolic, linolenic and isolinolenic acids Similar mixtures are found in other fruits and seeds.

Since glycerol is a trihydric alcohol, it would be possible for one or more of the three hydroxyls to react with the acid to form mono-, di- or tri-glycerides All these cases occur and, sometimes, one hydroxyl is replaced by one acid, and another hydroxyl by a different acid.

When the distribution of fats among the flowering plants is considered, they are found to be more widely distributed than the botanist is generally led to suppose

The following is a list of some of the plants especially rich in fats as reserve material in the fruits or seeds It represents only a selection of the better known genera, since many other plants have fatty seeds An approximate percentage of oil present in the fruit or seed is given.

Graminaceae Maize (*Zea Mays*) 4 %

Palmaceae. Oil Palm (*Elaeis guineensis*) 62 % Coconut Palm (*Cocos nucifera*) 65 %

Juglandaceae Walnut (*Juglans regia*) 52 %

Betulaceae Hazel (*Corylus Avellana*) 55 %.

Moraceae Hemp (*Cannabis sativa*) 33 %

Papaveraceae Opium Poppy (*Papaver somniferum*) 47 %.

Cruciferae Garden Cress (*Lepidium sativum*) 25 %. Black Mustard (*Sinapis nigra*) 20 %: White Mustard (*Sinapis alba*) 25 %: Colza (*Brassica rapa* var *oleifera*) 33 % Rape (*Brassica napus*) 42 %

Rosaceae Almond (*Prunus Amygdalus*) 42 % Peach (*P. Persica*) 35 % Cherry (*P Cerasus*) 35 % Plum (*P domestica*) 27 %.

Linaceae Flax (*Linum usitatissimum*) 20–40 %.

Euphorbiaceae. Castor-oil (*Ricinus communis*) 51 %.

Malvaceae. Cotton (*Gossypium herbaceum*) 24 %.

Sterculiaceae Cocoa (*Theobroma Cacao*) 54 %

Lecythidaceae Brazil Nut (*Bertholletia excelsa*) 68 %.

Oleaceae Olive (*Olea europaea*) 20–70 %. Ash (*Fraxinus excelsior*) 27 %

Rubiaceae Coffee (*Coffea arabica*) 12 %

Cucurbitaceae Pumpkin (*Cucurbita Pepo*) 41 %

Compositae· Sunflower (*Helianthus annuus*) 38 %

The conclusion must not be drawn from the above list that the seeds of the plants mentioned have exclusively fats as reserve materials. In many cases fat may be the chief reserve product, but in others it may be accompanied by either starch or protein or both.

Some of the best-known examples of fat-containing seeds which yield "oils" of great importance in commerce, medicine, etc, are *Ricinus* (castor oil), *Brassica* (colza oil), *Gossypium* (cotton-seed oil), *Cocos* (coconut oil), *Elaeis* (palm oil), *Olea* (olive oil)

In the plant the fats are present as globules in the cells of the fat-containing tissues

Plant fats may vary from liquids, through soft solids, to wax-like solids which generally have low melting-points. They float upon water in which they are insoluble. They are soluble in ether, petrol ether, benzene, chloroform, carbon tetrachloride, carbon bisulphide, etc some are soluble in alcohol With osmic acid fats give a black colour, and they turn red with Alkanet pigment which they take into solution

Expt 82 *Tests for fats* Weigh out 50 gms. of Linseed (*Linum usitatissimum*) and grind in a coffee-mill. Put the linseed meal into a flask, cover with ether, cork and allow the mixture to stand for 2 -12 hrs Filter off the ether into a flask, fit with a condenser and distil off the ether over an electric heater (If a heater is not available, distil from a water-bath of boiling water after the flame has been turned out.) When the bulk of the ether is distilled off, pour the residue into an evaporating dish on a water-bath and drive off the rest of the ether With the residue make the following tests in test-tubes.

(*a*) Try the solubilities of the oil in water, petrol ether, alcohol and chloroform. It is insoluble in water and alcohol, but soluble in petrol ether and chloroform

(*b*) Add a little 1 % solution of osmic acid A black colour is formed (This reaction is employed for the detection of fat in histological sections.)

(*c*) Add to the oil a small piece of Alkanet (*Anchusa officinalis*) root, and warm gently on a water-bath. The oil will be coloured red. Divide the oil into two portions in test-tubes To one add a little water, to the other alcohol. The coloured oil will rise to the surface of the water in one case, and sink below the alcohol in the other The Alkanet pigment being insoluble in both water and alcohol, these liquids remain uncoloured

Keep some of the linseed oil for Expt. 83

It is well known that the hydrocarbons of the unsaturated ethylene series C_nH_{2n} will combine directly with the halogens, chlorine, bromine and iodine to give additive compounds, thus:

$$C_2H_4 + Br_2 = \underset{\text{ethylene bromide}}{C_2H_4Br_2}$$

The acids of this series also behave in the same way, and since many plant fats contain members of the series, the fats will also combine with the halogens.

Expt. 83. *To show the presence of unsaturated groups in a fat.* To a little of the linseed extract add bromine water. Note the disappearance of the bromine and the formation of a solid product.

One of the most important chemical reactions of fats is that known as saponification. When a fat is heated with an alkaline hydroxide the following reaction takes place:

$$\begin{array}{l} C_{17}H_{35}CO\ O{-}CH_2 \\ C_{17}H_{35}CO\ O{-}CH \\ C_{17}H_{35}CO\ O{-}CH_2 \\ \text{tristearin} \end{array} + 3KOH = 3C_{17}H_{35}COOK + \underset{\text{glycerol}}{CH_2OH\ CHOH\ CH_2OH}$$

The potassium salt, potassium stearate, of the fatty acid, stearic acid, is termed a soap. The ordinary soaps used for washing are mixtures of such alkali salts of the various fatty acids occurring in vegetable and animal fats, and are manufactured on a large scale by saponifying fats with alkali. The soaps are soluble in water, so that when a fat is heated with a solution of caustic alkali, the final product is a solution of soap, glycerol and excess of alkali. The soap is insoluble in saturated salt (sodium chloride) solution, and when such a solution is added to the saponified mixture, the soap separates out and rises to the surface of the liquid. This process is known as "salting out." If the saponified mixture is allowed to cool without salting out, it sets to a jelly-like substance. When caustic potash is used for saponification and the product is allowed to set, a "soft" soap is formed. Hard soaps are prepared by using caustic soda and salting out.

The properties of soaps in solution are important. When a soap goes into solution, hydrolysis takes place to a certain extent with the formation of free fatty acid and free alkali. The free fatty acid then forms an acid salt with the unhydrolyzed soap. This acid salt gives rise to an opalescent solution and lowers the surface tension of the water with the result that a lather is readily formed.

The property of soaps of lowering surface tension is the reason for

their producing very stable emulsions when added to oil and water (see chapter on colloids, p 11)

Expt. 84. *Hydrolysis of fat with alkali* Take 12 Brazil nuts, the seeds of *Bertholletia* (Lecythidaceae) Crack the seed coats and pound the kernels in a mortar Put the pounded nut in a flask, cover it with ether, and allow the mixture to stand for 2–12 hrs Filter into a weighed or counterpoised flask and distil off the ether as in Expt 82 Weigh the oil roughly and add 4–5 times its weight of alcoholic caustic soda (prepared by dissolving caustic soda in about twice its weight of water and mixing the solution with twice its volume of alcohol) Heat on a water-bath until no oil can be detected when a drop of the mixture is let fall into a beaker of water Then add saturated sodium chloride solution The soaps will rise to the surface Allow the soaps to separate out for a time and then filter Press the soap dry with filter-paper, and test a portion to see that it will make a lather Neutralize the filtrate from the soap with hydrochloric acid and evaporate as nearly as possible to dryness on a water-bath Extract the residue with alcohol and filter Test the filtrate for glycerol by means of the following tests

(*a*) To a little of the solution add a few drops of copper sulphate solution and then some sodium hydroxide A blue solution is obtained owing to the fact that glycerol prevents the precipitation of cupric hydroxide

(*b*) Treat about 5 c c. of a 0 5 % solution of borax with sufficient of a 1 % solution of phenolphthalein to produce a well-marked red colour. Add some of the glycerol solution (which has first been made neutral by adding acid) drop by drop until the red colour just disappears Boil the solution : the colour returns. The reaction is probably explained thus. Sodium borate is slightly hydrolyzed in solution and boric acid, being a weak acid, is only feebly ionized, and therefore the solution is alkaline. On adding glycerol, glyceroboric acid (which is a strong acid) is formed and so the reaction changes to acid. On heating, the glyceroboric acid is hydrolyzed to glycerol and boric acid, and the solution again becomes alkaline

(*c*) Heat a drop or two with solid potassium hydrogen sulphate in a dry test-tube ; the pungent odour of acrolein (acrylic aldehyde) should be noted

$$C_3H_8O_3 = C_2H_3 \cdot CHO + 2H_2O.$$

In addition to Brazil nuts, the following material can also be used · endosperm of Coconut, ground linseed, almond kernels and shelled seeds of the Castor-oil plant (*Ricinus*) about 50 gms. should be taken in each case

Expt 85 *Reactions of soaps* (*a*) Take some of the soap which has been filtered off and shake up with water in a test-tube. A lather should be formed (*b*) Make a solution of a little of the soap in a test-tube and divide it into three parts To each add respectively a little barium chloride, calcium chloride and lead acetate solutions. The insoluble barium, calcium and lead salts will be precipitated. (The curd which is formed in the case of soap and hard water is the insoluble calcium salt) Thirdly, take the remainder of the soap and acidify it with dilute acid in an evaporating dish, and warm a little on a water-bath. The soap is decomposed and the fatty acids are set free and rise to the surface.

Expt. 86 *Reactions of fatty acids* (*a*) Try the solubilities in ether and alcohol of the acids from the previous experiment They are soluble (*b*) Shake an alcoholic solution of the fatty acids with dilute bromine water The colour of the bromine is discharged owing to the bromine forming additive compounds with the unsaturated acids

The question of the metabolism of fats in the plant is a very complicated one and has not yet been satisfactorily investigated. All plants may have the power of synthesizing fats, and a great number, as we have seen, contain large stores of these compounds in the tissues of the embryo, or endosperm, or both The point of interest is that of tracing the processes by which these fats are synthesized, and are again hydrolyzed and decomposed The products of decomposition may serve for the synthesis of other more vital compounds as the embryo develops, and before it is able to synthesize the initial carbohydrates, and to absorb the salts requisite for general plant metabolism

One fact seems fairly clear, namely that when fat-containing seeds germinate, an enzyme is present in the tissues which has the power of hydrolyzing fats with the formation of fatty acids and glycerol. Such enzymes are termed lipases

The lipase which has been most investigated is that which occurs in the seeds of the Castor-oil plant (*Ricinus communis*) It has been shown that if the germinating seeds are crushed and allowed to autolyze (p 19) in the presence of an antiseptic, the amount of fatty acid increases, whereas in a control experiment in which the enzyme has been destroyed by heat, no such increase takes place (Reynolds Green, 9, 10)

Investigation has shown the enzyme to be present also in the resting seed, but in an inactive condition as a so-called zymogen (Armstrong, 4, 5, 6, 7) The zymogen is considered to be a salt and, after acidification with weak acids, the salt is decomposed, and the enzyme becomes active After the preliminary treatment with acid, however, the enzyme is most active in neutral solution. The effect of acid on the zymogen may be demonstrated by autolyzing the crushed seed with a little dilute acetic acid, the increase of acidity will be found to be much greater than in the case of a control experiment in which acid has not been added.

It has not been found possible to extract the enzyme from the resting seed An active material can be obtained by digesting the residue, after extraction of the fat, with dilute acetic acid and finally washing with water This material can then be used for testing the hydrolytic power of the enzyme on various fats.

There is little doubt that lipase catalyzes the synthesis of fats as well as the hydrolysis, the reaction, in fact, has been carried out to a certain extent *in vitro*

Expt 87 *Demonstration of the existence of lipase in ungerminated* Ricinus *seeds*

A Remove the testas from about two dozen *Ricinus* seeds and pound the kernels up in a mortar Into three small flasks (*a*), (*b*) and (*c*), put the following

(*a*) 2 gms of pounded seed + 10 c c of water

(*b*) 2 gms of pounded seed + 10 c c of water + 2 c c of N/10 acetic acid.

(*c*) 2 gms of pounded seed + 10 c c of water + 2 c c of N/10 acetic acid, and boil well.

Add a few drops of chloroform to all three flasks, plug them with cotton-wool, and allow them to incubate for 12 hours at 37° C Then add 2 c c of N/10 acetic acid to flask (*a*), and 25 c c of alcohol to all three flasks Titrate the fatty acids present with N/10 alkali, using phenolphthalein as an indicator. A greater amount of fat should be hydrolyzed in (*b*) than in (*a*), and also slightly more in (*a*) than in (*c*) The addition of alcohol checks the hydrolytic dissociation of the soap formed on titration

B Pound up about 15 gms of *Ricinus* seeds which have been freed from their testas, and let the pounded mass stand with ether for 12 hrs. Then filter, wash with ether and dry the residue Weigh out three lots, of 2 gms each, of the fat-free meal and treat as follows

(*a*) Grind up the 2 gms of meal in a mortar with 16 c c of N/10 acetic acid (1 c 8 c c of acid to 1 gm of meal), and let it stand for about 15 minutes Then wash well with water to free from acid, and transfer the residue to a small flask Add 5 c c of castor oil, 2 c c. of water and a few drops of chloroform

(*b*) Treat the 2 gms of meal as in (*a*), but, after washing, and before transferring to the flask, boil well with a little distilled water Add 5 c c. of oil, 2 c c. of water and a few drops of chloroform

(*c*) Put the 2 gms. of meal into the flask without treatment and then add 5 c c of oil, 2 c c of water and a few drops of chloroform.

Incubate all three flasks for 12 hours, and then titrate with N/10 caustic soda, after addition of alcohol as in *A* A certain amount of acetic acid is always retained by the seed residue, and this is ascertained from the value for flask (*b*). Flask (*c*) will act as the control

Another question to be considered is the mode of synthesis in the plant of the complex fatty acids which form the components of the fats No conclusive work has been done in this direction, but many investigators have held the view that the fats arise from carbohydrates, notably the sugars. In fact, it has been shown that in *Paeonia* and *Ricinus*, as the seeds mature, carbohydrates disappear and fats are formed.

The sequence of events, however, in the synthesis of fatty acids from sugars is very obscure. If we examine the formulae, respectively, of a hexose

$$CH_2OH \cdot CHOH \cdot CHOH \cdot CHOH \cdot CHOH \cdot CHO$$

and a fatty acid, e g myristic acid

$H_3C—CH_2 \cdot CH_2\ CH_2\ CH_2 \cdot CH_2\ CH_2\ CH_2\ CH_2\ CH_2\ CH_2\ CH_2 \cdot COOH$

it is seen that three main changes are concerned in the synthesis of such a fatty acid from sugar, i e reduction of the hydroxyl groups of the sugar, conversion of the aldehyde group into an acid group, and finally the condensation or linking together of chains of carbon atoms. An interesting fact in connexion with this point is that all naturally occurring fatty acids have a straight, and not a branched, carbon chain It has been suggested (Smedley, etc, 11–13) that acetaldehyde and a ketonic acid, pyruvic acid, may be formed from sugar. By condensation of aldehyde and acid, another aldehyde is formed with two more carbon atoms By repetition of the process, with final reduction, fatty acids with straight chains are produced.

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CHAPTER VII

AROMATIC COMPOUNDS AND OXIDIZING ENZYMES

THE aromatic compounds may be defined as substances containing the benzene carbon ring or a similar ring A very great number occur among the higher plants but of these many are restricted in distribution, and may only be found in a few genera or even in one genus. others, on the other hand, are widely distributed At present our knowledge of the part they play in general plant metabolism is obscure.

The more widely distributed aromatic plant products may be grouped as

1 The phenols, and their derivatives.

2 The aromatic alcohols, aldehydes and acids (including the tannins), and their derivatives.

3 The flavone, flavonol and xanthone pigments, known as the soluble yellow colouring matters.

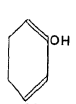
4 The anthocyan pigments, known as the soluble red, purple and blue colouring matters.

In connexion with the aromatic compounds it should be noted that many of them contain hydroxyl groups, and one or more of these groups may be replaced by the glucose residue, $C_6H_{11}O_5$—, with elimination of water and the formation of a glucoside, in the way already described (see p 48). The majority of such compounds are sometimes classed together as a group—the glucosides—regardless of the special nature of the substance to which the glucose is attached (this course has been followed to some extent in Chapter IX with compounds, the chief interest of which lies in their glucosidal nature) In treating of the aromatic substances in the following pages, mention will be made when they occur as glucosides, this combination being in these cases only a subsidiary point in their structure.

The various groups of aromatic substances will now be considered in detail.

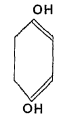
PHENOLS.

There are three dihydric phenols, resorcinol, catechol and hydroquinone, but of these only the last is known to exist in the free state in plants. The two former frequently occur as constituents of complex plant products, and may be obtained on decomposition of such complexes by fusion with strong alkali, etc



Resorcinol

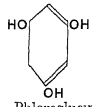
Catechol



Hydroquinone

Hydroquinone has been found in the free state in the leaves and flowers of the Cranberry (*Vaccinium Vitis-Idaea*). As a glucoside, known as arbutin, it occurs in many of the Ericaceae (see also p. 151).

Phloroglucin is the only member of the trihydroxy phenols found uncombined in plants It is very widely distributed in the combined state in various complex substances (Waage, 27)

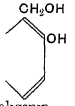


Phloroglucin

Aromatic Alcohols and Aldehydes

The following are some of the better known compounds of this group·

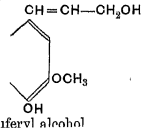
Saligenin, or salicylic alcohol, in the form of the glucoside, salicin, occurs in the bark of certain species of Willow (*Salix*), and in the flower-buds of the Meadow-sweet (*Spiraea Ulmaria*) Salicin is hydrolyzed by an enzyme contained in the plant in which it occurs, into saligenin and glucose (see also p. 152)



Saligenin

Salicylic aldehyde occurs in species of *Spiraea* and other plants.

Coniferyl alcohol, as a glucoside, coniferin, is found in various conifers and also in Asparagus (*Asparagus officinalis*) Coniferin is hydrolyzed by dilute acids or by enzymes (emulsin) into coniferyl alcohol and glucose (see also p 151).



Coniferyl alcohol

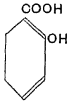
Coniferyl alcohol when oxidized yields the aldehyde, vanillin (so much used for flavouring), which occurs in the fruits of the Orchid (*Vanilla planifolia*).

Aromatic Acids

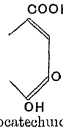
Of this group the following are some of the best known representatives

Salicylic acid is a monohydroxybenzoic acid It occurs both in the form of esters and in the free state in various plants

Protocatechuic acid is a dihydroxybenzoic acid. It has been found in the free state in a few plants, but is more widely distributed as a constituent of many plant products As will be shown later it forms the basis of one of the series of tannins.

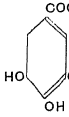


Salicylic acid



Protocatechuic acid

Gallic acid is a trihydroxybenzoic acid ·



It occurs free in gall-nuts, in tea, wine, the bark of some trees and in various other plants It forms a constituent of many tannins It is a crystalline substance not very readily soluble in cold but more soluble in hot water. In alkaline solution it rapidly absorbs oxygen from the air and becomes brown in colour

Expt 88 *The extraction and reactions of gallic acid* Take 100 gms. of tea, dry in a steam oven and grind in a mortar Put the powder into a flask and cover well with ether The preliminary drying and grinding can be omitted, but if carried out will make the extraction more complete. After at least 24 hrs filter off the extract, and either distil or evaporate off the ether The ether will be coloured deep green by the chlorophyll present in the dried leaves, and a green residue will be left. Add about 20 c.c. of distilled water to the residue, heat to boiling and filter Heating is necessary because the gallic acid is only sparingly soluble in cold water Keep the residue for Expt 91 With the filtrate make the following tests; for (*a*), (*b*) and (*c*) dilute a few drops of the filtrate in a porcelain dish

(*a*) Add a drop of ferric chloride solution. A blue-black coloration is given.

(*b*) Add a drop or two of iodine solution. A transient red colour appears

(*c*) Add a drop or two of lime water. A reddish or blue coloration will be given.

(*d*) To a few c.c. of the filtrate in a porcelain dish add a little lead acetate solution. A precipitate is formed which turns red on addition of caustic potash solution, and dissolves to a red solution with excess of potash

(*e*) To a few c.c. of the filtrate in a test-tube add a little potassium cyanide solution. A pink colour appears, but disappears on standing. On shaking with air it reappears

(*f*) To a few c.c. of the filtrate in a test-tube add a few drops of 10 % gelatine solution. No precipitate is formed

(*g*) To a few c.c. of the filtrate in a test-tube add a little lead nitrate solution. No precipitate is formed

TANNINS.

This is a large group of substances, many of which are of complex composition. They arise in the plant from simpler compounds, such as protocatechuic, gallic and ellagic acids. Their formation takes place in various ways, either by condensation, accompanied by elimination of water, or by oxidation, or both; there may also be condensation with other aromatic complexes.

The tannins are widely distributed in the higher plants and, although no very systematic investigation has been made, it is obvious that some plants are rich in these substances, others poor, and others, again, apparently entirely without them. The tannins generally occur in solution in the cells of tissues of the root, stem, leaf, fruit, seed and flowers; sometimes they are confined to special cells, tannin-sacs, but after the death of the cell, the cell-walls of the dead tissue become impregnated with the tannin. In tannin-producing plants, the tannin is generally found throughout the plant, and it probably tends to accumulate in permanent or dead tissues, such as the bark (dead cortex and cork), woody tissue, underground stems, etc.

Tannins appear to be more frequent in woody than in herbaceous plants, though in the latter they naturally only accumulate in the persistent underground stems and root-stocks. In annuals, also, tannins seem to be more rare: this may be due to the fact that in a short-lived plant, comparatively little tannin is formed and is not so readily detected as in the tissues of a perennial.

In certain plants which are highly tannin-producing and are also woody perennials, the bark becomes very rich in tannins. These barks are consequently of considerable commercial importance for tanning of

leather As examples may be taken species of *Caesalpinia*, Spanish Chestnut (*Castanea*), *Eucalyptus*, Oak (*Quercus*), Mangrove (*Rhizophora*), Sumac (*Rhus*) Tannins also occur in quantity in galls, especially on species of *Quercus*.

As a class, the tannins are non-crystalline and exist in the colloidal state in solution. They have a bitter astringent taste. They have certain properties and reactions in common, i e they precipitate gelatine from solution, are themselves precipitated from solution by potassium bichromate, and give either blue or green colorations with solutions of iron salts. Many tannins occur as glucosides but this is by no means always the case

It is possible to classify the tannins into two groups according as to whether they are complexes derived from protocatechuic acid or gallic acid

1 The pyrogallol tannins. These give a dark blue colour with ferric chloride solution, and no precipitate with bromine water.

2. The catechol tannins. These give a greenish-black colour with iron salts, and a precipitate with bromine water

Expt 89 *Reactions of tannins* Take three oak galls (the brown galls formed by species of *Cynips* on the Common Oak) and pound them finely in a mortar Boil up the powder well with a small amount of water in an evaporating basin and let stand for a short time. Then filter The filtrate will contain tannin together with impurities Make the following tests with the extract.

(*a*) Put 2 c c of the tannin extract into a small evaporating dish, dilute with water, and add a drop or two of ferric chloride solution A deep blue-black colour is produced

(*b*) Put 2 or 3 drops of the tannin extract into a small evaporating dish, and dilute with water add a little dilute ammonia and then a few drops of a dilute solution of potassium ferricyanide solution. A red coloration will appear

(*c*) To 5 c c of the tannin solution in a test-tube add some strong potassium dichromate solution The tannin will be precipitated.

(*d*) To about 5 c c of the tannin extract in a test-tube add a little lead acetate solution The tannin will be precipitated.

(*e*) Melt a little of a 10 % solution of gelatine by warming gently and then pour drop by drop into a test-tube half full of tannin extract. The gelatine will be precipitated

For the above tests, in addition to galls, the bark stripped from two to three year old twigs of *Quercus* may also be used, and will give the same reactions The bark should be cut into small pieces for extraction.

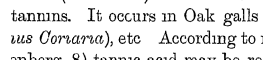
It should be noted that although many tannins give the above reactions, it does not necessarily follow that all tannins will give all the reactions.

To demonstrate the existence of pyrogallol and catechol tannins The
a pyrogallol tannin which gives a blue reaction with iron salts has
ted in the last experiment on the Oak galls and the bark from Oak twigs
the Sumac (*Rhus Coriaria*) and the fruit pericarp, leaves and bark of the
nut (*Castanea vulgaris*) may be used as additional material for pyrogallol

on-greening tannin strip off the outer bark from two to three year old
Horse Chestnut (*Aesculus Hippocastanum*) Cut or tear the bark into
and boil *well* with a little water in an evaporating dish Filter and test
vith ferric chloride solution as in Expt 89 A green coloration will be
-greening tannins may also be extracted from the bark of twigs of the
dans regia) and of the Larch (*Larix europaea*)
se of both classes of tannins, in addition to the ferric chloride reaction,
Expt 89 (*c*) and (*e*) should also be made on the extracts, in order to
presence of tannin, since other substances, such as flavones, may give a
with iron salts (see p 94)

of the individual tannins will now be considered

annic (or tannic) acid is one of the most important of the
tannins. It occurs in Oak galls and Oak wood, in tea, in the
us Coriaria), etc According to recent investigations (Fischer
enberg, 8) tannic acid may be regarded as a compound of one
f glucose with five molecules of digallic acid in which five
of the sugar are esterified by five molecules of acid·



acid is an almost colourless amorphous substance. It has an
taste, is soluble in water and alcohol, only slightly soluble in
insoluble in chloroform It is decomposed, by boiling with
hloric acid, into gallic acid

Extraction and reactions of tannic (or gallotannic) acid. By a crude
ution of gallotannic acid can be obtained from tea. About 5 gms of the
· the extraction with ether in Expt 88, is again extracted with ether
: which will remove all but traces of gallic acid. Boil up the residue from
little water and filter With the filtrate make the following tests which
between gallic and gallotannic acid
bout 10 c c add a little 10 % gelatine The gelatine is precipitated
little of the filtrate add a few drops of lead nitrate solution. The tannic
ntated
aning tests are given in common with gallic acid. If the extract is too
ute with water
: a few drops of the filtrate with water in a porcelain dish and add a
: chloride solution A blue-black colour is given

(*d*) Dilute a few drops of the filtrate with water in a porcelain dish and add a drop or two of iodine solution. A transient red colour is formed.

(*e*) To a little of the filtrate in a test-tube add a few drops of potassium cyanide solution. A reddish-brown colour is formed which changes to brown but becomes red again on shaking with air.

In addition to tannic acid, a great many other tannins are known, but their constitution is obscure.

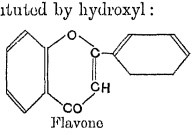
Expt 92. *To demonstrate that in tannin-containing plants the tannin may be also present in the leaves.* Take about two dozen leaves of the Common Oak (*Quercus Robur*) and pound them in a mortar. Then boil the crushed mass in an evaporating dish with a little water. Filter, and with the filtrate make the tests for tannin. Leaves of other trees also may be used, e.g. the Wig Tree (*Rhus Cotinus*), Sweet Chestnut (*Castanea vulgaris*).

Expt 93. *To demonstrate that tannins may be present in herbaceous as well as woody plants.* Extract some leaves, as in the last experiment, of Scarlet Geranium (*Pelargonium zonale*) and test for tannin.

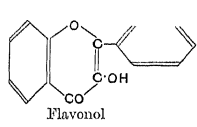
Expt 94. *To demonstrate that tannins may be present in petals and fruits, in addition to other parts of the plant.* Extract and test for tannins as in the last experiment, using petals of *Pelargonium zonale*, Common Paeony (*Paeonia officinalis*) or Rose (any garden variety), inflorescence of Flowering Currant (*Ribes sanguineum*), flowers of Horse Chestnut (*Aesculus Hippocastanum*) or pericarp of Sweet Chestnut (*Castanea*).

The Flavone and Flavonol Pigments.

These yellow colouring matters are very widely distributed in the higher plants (Shibata, Nagai and Kishida, 26). They are derived from the mother substances, flavone and flavonol, the latter only differing from the former in having the hydrogen in the central γ-pyrone ring substituted by hydroxyl:



Flavone



Flavonol

The naturally occurring pigments, however, have additional hydrogen atoms replaced by hydroxyl groups, that is they are hydroxyflavones and flavonols, and the various members differ among each other in the number and position of these hydroxyl groups. Some of the members are widely distributed, others less so. Quite often more than one representative is present in a plant.

The flavone and flavonol pigments are yellow crystalline substances, and as members of a class they have similar properties. They occur in the plant most frequently as glucosides, one or more of the hydroxyl groups being replaced by glucose, or, sometimes, by some other hexose, or pentose. In the condition of glucosides, they are much less coloured than in the free state, and, being present in the cell-sap in very dilute solution, they do not produce any colour effect, especially in tissues containing chlorophyll. Occasionally they give a yellow colour to tissues, as in the rather rare case of some yellow flowers (*Antirrhinum*) where colour is due to soluble yellow pigment.

In the glucosidal state, the flavone and flavonol pigments are, as a rule, readily soluble in water and alcohol, but not in ether In the non-glucosidal state they are, as a rule, readily soluble in alcohol, somewhat soluble in ether, but soluble with difficulty in water

The flavone and flavonol pigments can be easily detected in any tissue by the fact that they give an intense yellow colour with alkalies (Wheldale, 29). If plant tissues be held over ammonia vapour, they turn bright yellow, showing the presence of flavone or flavonol pigments the colour disappears again on neutralization with acids (The reaction is especially well seen in tissues free from chlorophyll, such as white flowers) This reaction will be found to be almost universal, showing how wide is their distribution With iron salts, solutions of the pigments give green or brown colorations With lead, insoluble salts are formed Several of the members are powerful yellow dyes, and hence some plants in which they occur, such as Ling (*Erica cinerea*), Dyer's Weld or Rocket (*Reseda luteola*), have been used for dyeing purposes The value of these colouring matters as dyes has led to their chemical investigation, and as a result the constitution, etc, of the hydroxy-flavones and flavonols is well established

Expt 95 *Demonstration of the presence of flavone or flavonol pigments in tissues without chlorophyll.* Take flowers of any of the undermentioned species and put them in a flask with a few drops of ammonia They will rapidly turn yellow owing to the formation of the intensely yellow salt of the flavone or flavonol pigments present in the cell-sap. If the flowers are next treated with acid the yellow colour will disappear

Also make an extract of some of the flowers with a little boiling water. Filter, cool and add the following reagents

(*a*) A little alkali A yellow colour is produced

(*b*) A little ferric chloride solution. Either a green or brown coloration is produced.

(*c*) A little basic lead acetate solution A yellow precipitate of the lead salt of the flavone or flavonol pigment is formed

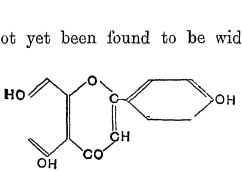
The flowers of the following species can be used Snowdrop (*Galanthus nivalis*), Narcissus (*Narcissus poeticus*), white variety of Lilac (*Syringa vulgaris*), Hawthorn (*Crataegus Oxyacantha*), White Lily (*Lilium candidum*), white var of Phlox, double white Pink, white Stock (*Matthiola*) etc, etc, in fact almost any species with white flowers or a white variety

Expt 96 *Demonstration of the presence of flavone or flavonol pigments in tissues containing chlorophyll.* Make a hot water extract of the leaves of any of the under-mentioned species Make with it the same tests as in the previous experiment.

The following plants may be used Snowdrop (*Galanthus nivalis*), Dock (*Rumex obtusifolius*), Goutweed (*Aegopodium Podagraria*), Dandelion (*Taraxacum officinale*), Violet (*Viola odorata*), Ribwort Plantain (*Plantago lanceolata*), Elder (*Sambucus nigra*).

The most important *flavone* pigments are apigenin, chrysin and luteolin

Apigenin has not yet been found to be widely distributed. Its formula is



It occurs in the Parsley (*Carum Petroselinum*) (Perkin, 18) and in the flowers of the ivory-white variety of Snapdragon (*Antirrhinum majus*) (Wheldale and Bassett, 30).

Expt 97 *Extraction of apiin, the glucoside of apigenin, from the Parsley* (Carum Petroselinum). Take some Parsley leaves and boil in as little water as possible Filter off the extract and make the following tests for apigenin:

(*a*) Add alkali A lemon yellow coloration is given.

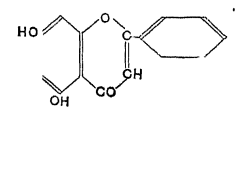
(*b*) Add basic lead acetate solution A lemon yellow precipitate is formed

(*c*) Add ferric chloride solution A brown colour is produced.

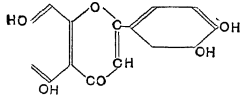
(*d*) Add ferrous sulphate solution A reddish-brown colour is produced.

Apiin frequently separates out in a gelatinous condition from aqueous and dilute alcoholic solutions.

Chrysin is a flavone occurring in the buds of various species of Poplar (*Populus*) It has the formula



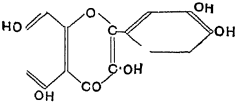
Luteolin does not appear to be widely distributed, though possibly it occurs in many plants in which it has not yet been demonstrated. Its formula is represented as



It occurs in the Dyer's Weld or Wild Mignonette (*Reseda luteola*) (Perkin, 17), Dyer's Greenweed or Broom (*Genista tinctoria*) (Perkin, 23) and in the yellow variety of flowers of the Snapdragon (*Antirrhinum majus*) (Wheldale and Bassett, 32) It has been much used as a yellow dye hence the names of the first two plants (Perkin and Horsfall, 20)

The most important *flavonol* pigments are quercetin, kaempferol, myricetin and fisetin

Quercetin is apparently one of the most widely distributed of the whole group of yellow pigments, and has the formula



It occurs, either free, or combined with various sugars (glucose, rhamnose) as glucosides, in many plants, as for instance the following: in the bark of species of Oak (*Quercus*), in berries of species of Buckthorn (*Rhamnus*), in flowers of Wallflower (*Cheiranthus Cheiri*), Hawthorn (*Crataegus Oxyacantha*) (Perkin and Hummel, 22), Pansy (*Viola tricolor*) (Perkin, 19) and species of Narcissus in leaves of Ling (*Calluna erica*) (Perkin, 23), and the outer scale leaves of Onion bulbs (Perkin and Hummel, 21)

Expt 98. *Preparation of a glucoside of quercetin from Narcissus flowers.* The species of Narcissus which can be used are *N Tazetta*, *N incomparabilis*, and any of the common yellow trumpet varieties Pound about 50 flowers in a mortar and then extract in a flask with boiling alcohol Filter off the alcoholic extract and evaporate to dryness, then add a little water and ether and transfer the whole to a separating funnel The ether takes up the plastid pigments, but at the plane of separation of the liquids, the glucoside separates out as a crystalline deposit This can be filtered off; with a dilute solution in alcohol make the following tests

(*a*) Add a little alkali The yellow colour is intensified, but the intensification disappears on adding acid

(*b*) Add a little lead acetate solution An orange precipitate of the lead salt formed

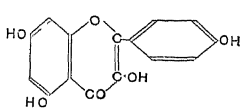
(*c*) Add a little ferric chloride solution A green coloration is produced

(*d*) Heat some of the alcoholic solution on a water-bath, acidify with strong hydrochloric acid and add zinc dust A magenta colour is produced (see p 106)

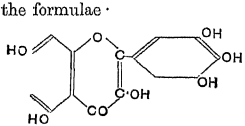
Expt 99 *Preparation of crude quercetin from Onion skins* (Perkin, 21) Take about 50 gms of the brown outer skins of onions and boil with 900 c c of water for an hour Then filter and allow the filtrate to stand for 24 hrs A brownish-yellow deposit is formed which is crude quercetin Filter this off and dissolve in 75% alcohol and allow to evaporate slowly Quercetin will be deposited With a solution in dilute alcohol make the same tests as in the last experiment

Expt 100 *Preparation of a quercetin glucoside from Wallflower* (Cheiranthus Cheiri) *flowers* (Perkin and Hummel, 22) Take 20 gms of petals of flowers of either the brown or yellow variety and drop them into boiling alcohol in a flask Filter and evaporate the extract to dryness on a water-bath Dissolve the residue in water and add ether In the case of the yellow variety the yellow plastid pigments are taken up by the ether, and the quercetin glucoside partly crystallizes out from the water as in Expt 98 In the case of the brown variety both quercetin glucoside and anthocyan pigment are present as well as plastid pigments The two former go into solution in the water and the glucoside in time crystallizes out In either case the glucoside can be filtered off and tested as in the previous experiment A positive result will be given in each case.

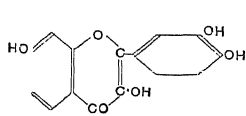
✓ **Kaempferol** occurs in the flowers of a species of Larkspur (*Delphinium consolida*) (Perkin and Wilkinson, 25) and *Prunus* (Perkin and Phipps, 24) and in the leaves or flowers of several other plants. It has the formula



✓ **Myricetin** and **fisetin** are two other flavones which have been found in species of Sumac (*Rhus*) and other plants. They have respectively the formulae·



Myricetin



Fisetin

The Anthocyan Pigments.

These pigments are the substances to which practically all the blue, purple and red colours of flowers, fruits, leaves and stems are due (Wheldale, 3) They occur in solution in the cell-sap and are very widely distributed, it being the exception to find a plant in which they are not produced As members of a group, they have similar properties, but differ somewhat among themselves, the relationships between them being much the same as those between the various flavone and flavonol pigments They occur in solution in the cell-sap but occasionally they crystallize out in the cell They are present in the plant in the form of glucosides, and in this condition they are known as *anthocyanins*, as glucosides they are readily soluble in water and as a rule in alcohol [except blue Columbine (*Aquilegia*), Cornflower (*Centaurea Cyanus*) and some others] but are insoluble in ether and chloroform The glucosides are hydrolyzed by boiling with dilute acids, and the resulting products, which are non-glucosidal, are termed *anthocyanidins* (Willstätter and Everest, 35) The latter, in the form of chlorides, are insoluble in ether, but are generally soluble in water and alcohol The anthocyanins can be distinguished from the anthocyanidins in solution by the addition of amyl alcohol after acidification with sulphuric acid. The anthocyanidins pass over into the amyl alcohol, the anthocyanins do not. The anthocyanins and anthocyanidins themselves (with one exception) have not yet been crystallized, but of both classes crystalline derivatives with acids have been obtained (Willstatter and Everest, 35).

In considering the reactions of anthocyan pigments the difference between those given by crude extracts and those of the isolated and purified substances must be borne in mind. With acids the anthocyan pigments give a red colour with alkalies they give, as a rule, a blue or violet colour when pure, but if flavone or flavonol pigments are present (as may be the case in a crude extract) they give a green colour, due to mixture of blue and yellow. In solution in neutral alcohol and water many anthocyan pigments lose colour, and this is said to be due to the conversion of the pigment into a colourless isomer which also gives a yellow colour with alkalies (Willstatter and Everest, 35), hence even a solution of a pure anthocyan pigment may give a green coloration with alkali due to mixture of blue and yellow. The isomerization can be prevented or lessened by addition of acids or of neutral salts which form protective addition compounds with the pigment With lead acetate

anthocyan pigments give insoluble lead salts, blue if the pigment is pure, or green, as in the case of alkalies, if it is mixed with flavone or flavonol pigments, or the colourless isomer

When anthocyan pigments are treated with nascent hydrogen, the colour disappears but returns again on exposure to air It is not known what reaction takes place

Expt 101 *The reactions of anthocyanins and anthocyanidins* Extract petals of the plants mentioned below with boiling alcohol in a flask Note that the anthocyan colour may disappear in the alcoholic extract Filter off some of the alcoholic extract and make the following tests (*a*) and (*b*) with it

(*a*) Add a little acid and note the bright red colour

(*b*) Add a little alkali and note the green colour

The decolorized petals, after filtering off the extract, should be warmed with a little water in an evaporating dish The colour is brought back if pigment is still retained by them

Evaporate the remainder of the alcoholic extract to dryness and note that the anthocyan colour returns Dissolve the residue in water and continue the following tests, taking a little of the solution in each case

(*c*) Add ether and shake The anthocyan pigment is not soluble in ether.

(*d*) Add acid A bright red colour is produced

(*e*) Add alkali A bluish-green or green colour is produced which may pass to yellow

(*f*) Add basic or normal lead acetate solution. A bluish-green or green precipitate is produced

(*g*) Add a little sulphuric acid and then amyl alcohol and shake the latter does not take up any of the red colour, indicating that the pigment is in the anthocyanin (glucosidal) state

(*h*) Heat a little of the solution on a water-bath with dilute sulphuric acid and then cool and add amyl alcohol The colour will pass into the amyl alcohol, indicating that the pigment is now in the anthocyanidin (non-glucosidal) state.

(*i*) Acidify a little of the solution with hydrochloric acid and add small quantities of zinc dust. The colour disappears Filter off the solution and note that the colour rapidly returns again

For the above reactions it is suggested that the following flowers be used as material magenta Snapdragon (*Antirrhinum majus*), brown Wallflower (*Cheiranthus Cheiri*), crimson Paeony (*Paeonia officinalis*), magenta "Cabbage" Rose, Violet (*Viola odorata*), but the majority of coloured flowers will serve equally well

Though the above represent the reactions and solubilities given by the greater number of anthocyan pigments, it will be found that all are not alike in these respects

Expt. 102. *Demonstration that anthocyanins may be insoluble in alcohol but soluble in water.* Extract petals of any of the species mentioned below with boiling alcohol and note that they do not lose their colour. It will be found that the pigments are either completely or largely insoluble in alcohol, but are soluble in water Test the

water extract as in Expt 101 (*c*)–(*i*) Also take equal quantities of the water extract in two evaporating dishes To one add sodium chloride Note (as mentioned above, see p 98) that the colour fades less rapidly from the extract containing the salt The flowers of the following species can be used . blue Larkspur (*Delphinium*), Cornflower (*Centaurea Cyanus*), blue Columbine (*Aquilegia*)

There is a small group of plants belonging to some allied natural orders, of which the anthocyan pigments give chemical reactions still more different from the general type already described, though they nevertheless resemble each other Such, for instance, are the pigments of various genera of the Chenopodiaceae [Beet (*Beta*), Orache (*Atriplex*)], Amarantaceae (*Amaranthus* and other genera), Phytolaccaceae (*Phytolacca*) and Portulacaceae (*Portulaca*) These anthocyan pigments are insoluble in alcohol but soluble in water they give a violet colour with acids, red to yellow with alkalies, and a red precipitate with basic lead acetate

Expt 103 *Reactions of the Beet-root* (Beta vulgaris) *pigment* Take some Beet-root leaves, tear them into small pieces and put them into alcohol Allow the leaves to stand for some time and note that the chlorophyll is extracted but the red pigment is insoluble Then pour off the alcohol and add water the red pigment goes into solution Filter off the solution and make the following tests

(*a*) Add acid The pigment turns violet
(*b*) Add alkali The pigment becomes redder and finally turns yellow
(*c*) Add basic lead acetate A red precipitate is formed
(*d*) Acidify with hydrochloric acid and add zinc dust The colour disappears, but on filtering off from the zinc it does not return again.

Anthocyan pigments may also occur in leaves, and this is very obvious in red-leaved varieties of various species such as the Copper Beech, the Red-leaved Hazel, etc

Expt 104 *Extraction of anthocyan pigment from the Red-leaved Hazel* Extract some leaves of the Blood Hazel (*Corylus Avellana* var. *rubra*) with alcohol Filter off and evaporate the solution to dryness Add water Pour a little of the crude mixture in the dish into a test-tube and add ether. There will be a separation into a green ethereal layer containing chlorophyll, and a lower water layer containing anthocyan pigment. Filter the extract remaining in the dish and with the filtrate make the tests already given in Expt 101 (*c*)–(*i*)

The leaves of the Copper Beech (*Fagus sylvatica* var *purpurea*) can also be used

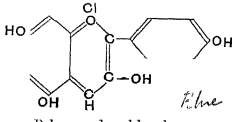
In many flowers, the cells of the corolla may contain yellow plastid (see p 39) in addition to anthocyan pigments. The colour of the petals is in these cases the result of the combination of the two, and is usually some shade of brown, crimson or orange-red, as in the brown-flowered variety of Wallflower (*Cheiranthus Cheiri*)

Expt 105 *Demonstration of the presence of anthocyan and plastid pigments together in petals (see also Expt* 100) Extract petals of the brown-flowered variety of Wallflower with alcohol Filter, and evaporate the extract to dryness Take up with water and add ether Pour the mixture into a separating funnel The plastid pigment will pass into solution in the ether, and the anthocyan pigment will remain in the water Test the aqueous solution as in Expt 101 (*c*)–(*i*)

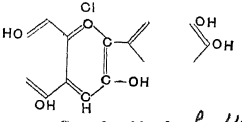
The following may be used as material ray florets of bronze or crimson *Chrysanthemum*, ray florets of *Gaillardia*, and orange-red flowers of Nasturtium (*Tropaeolum majus*)

Anthocyanins and anthocyanidins have been isolated from various species The pigments themselves with one exception have not been obtained in the crystalline state, but crystalline compounds with acids have been prepared both of the glucosidal and non-glucosidal forms.

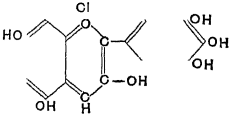
All the pigments so far described appear to be derived from three fundamental compounds, pelargonidin, cyanidin and delphinidin, of which the chlorides are represented thus·



Pelargonidin chloride

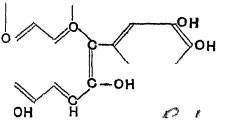


Cyanidin chloride



Delphinidin chloride

It has been suggested, at least in the case of cyanidin, the pigment of the Cornflower (*Centaurea Cyanus*), that the pigment itself is a neutral substance, purple in colour and of the following structure (Willstatter, 33, 36).



Further, that the blue pigment of the flower is the potassium salt of the purple, and the red acid salt, cyanidin chloride, depicted above, is a so-called oxonium compound of the purple

Pelargonidin, moreover, has been prepared synthetically (Willstatter and Zechmeister, 45).

The above three pigments, either as glucosides or in the form of methylated derivatives, are found in a number of plants which are listed below (Willstatter, etc, 33–46) The sugar residues or methyl groups may, of course, occupy different positions, thus giving rise to isomers.

Pelargonidin.

Callistephin	Monoglucoside of pelargonidin	Flowers of Aster (*Callistephus chinensis*)
Pelargonin	Diglucoside of pelargonidin	Flowers of Scarlet Geranium (*Pelargonium zonale*), pink var of Cornflower (*Centaurea Cyanus*) and certain vars of Dahlia (*D variabilis*)

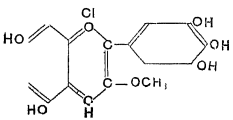
Cyanidin.

Asterin	Monoglucoside of cyanidin	Flowers of Aster (*Callistephus chinensis*)
Chrysanthemin	Monoglucoside of cyanidin	Flowers of Chrysanthemum (*C. indicum*)
Idaein	Monogalactoside of cyanidin	Fruit of Cranberry (*Vaccinium Vitis-Idaea*)
Cyanin	Diglucoside of cyanidin	Flowers of Cornflower (*Centaurea Cyanus*), *Rosa gallica* and certain vars of Dahlia (*D variabilis*)
Mekocyanin	Diglucoside of cyanidin	Flowers of Poppy (*Papaver Rhoeas*)
Keracyanin	Rhamnoglucoside of cyanidin	Fruit of Cherry (*Prunus Cerasus*)
Peonin	Diglucoside of peonidin (cyanidin monoethyl ether)	Flowers of Paeony (*Paeonia officinalis*)

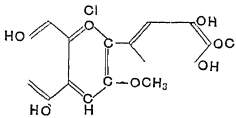
Delphinidin.

Violanin	Rhamnoglucoside of delphinidin	Flowers of Pansy (*Viola tricolor*) .
Delphinin	Diglucoside of delphinidin + *p*-hydroxybenzoic acid	Flowers of Larkspur (*Delphinium consolida*)
Ampelopsin	Monoglucoside of ampelopsidin (delphinidin monomethyl ether)	Fruit of Virginian Creeper (*Ampelopsis quinquefolia*)
Myrtillin	Monoglucoside of myrtillidin (delphinidin monomethyl ether)	Fruit of Bilberry (*Vaccinium Myrtillus*)
Althaein	Monoglucoside of myrtillidin	Flowers of deep purple var. of Hollyhock (*Althaea rosea*)
Petunin	Diglucoside of petunidin (delphinidin monomethyl ether)	Flowers of Petunia (*P violacea*)
Malvin	Diglucoside of malvidin (delphinidin dimethyl ether)	Flowers of Mallow (*Malva sylvestris*)
Oenin	Monoglucoside of oenidin (delphinidin dimethyl ether)	Fruit of Grape (*Vitis vinifera*)

Of the methylated compounds, myrtillidin and oenidin may be represented thus



Myrtillidin



Oenidin

Expt 106 *Preparation and reactions of pelargonin chloride* Extract the flowers from two or three large bosses of the Scarlet Geranium (*Pelargonium zonale*) in a flask with hot alcohol Filter off and concentrate on a water-bath Then pour the hot concentrated solution into about half its volume of strong hydrochloric acid On cooling, a crystalline precipitate of pelargonin chloride separates out Examine under the microscope and note that it consists of sheaves and rosettes of needles Filter off the crystals, take up in water and make the following experiments with the solution

(*a*) Add alkali A deep blue-violet colour is produced

(*b*) Take two equal quantities of solution in two evaporating dishes To one add as quickly as possible some solid sodium chloride The colour in the solution without salt will rapidly fade owing to the formation of the colourless isomer in neutral solution this change is prevented to a considerable extent in the solution containing salt owing to the formation of an addition compound of the pelargonin with the sodium chloride which prevents isomerization (see p 98) To portions of the water solution (without sodium chloride) which has lost its colour add respectively acid and alkali The red colour returns with acid owing to the formation of the red acid oxonium salt: with alkali a greenish-yellow colour will be produced due to the formation of the salt of the colourless isomer If alkali is added to the portion of the pigment solution containing the sodium chloride, it will be found that it still gives a violet colour.

(*c*) Add sulphuric acid and amyl alcohol The alcohol does not take up the colour Add amyl alcohol after acidifying another portion of the solution with sulphuric acid and heating on a water-bath The alcohol now abstracts some of the colour. This shows that the glucoside pelargonin exists in the first case, but is decomposed into the non-glucosidal pelargonidin after heating with acid

(*d*) Acidify with hydrochloric acid and add zinc dust. the colour disappears and returns again after filtering

Expt 107 *Preparation of the acetic acid salt of pelargonin.* Make an alcoholic extract of petals as in Expt 106. Evaporate down and pour into glacial acetic acid instead of hydrochloric acid The crystals of the salt formed are smaller and more purple in colour than those of the chloride.

Expt. 108 *Preparation and reactions of crude peonin chloride* Extract the petals of one or two flowers of the Crimson Paeony (*Paeonia officinalis*) with 95–98 % alcohol. Filter and evaporate nearly to dryness Then add some methyl alcohol and

pour into a little strong hydrochloric acid Then add ether to the mixture in a separating funnel A crude precipitate of peonin chloride will separate out after a time, which may be more or less crystalline Filter off this precipitate, take up in water and make the following experiments

(*a*) Take two equal quantities of the solution in two evaporating dishes To one add solid sodium chloride as in Expt 106 (*b*) Then neutralize both portions carefully with very dilute sodium carbonate solution until the colour changes slightly to purple The colour will fade more rapidly in the solution without sodium chloride on account of the formation of a colourless isomer, as in the case of pelargonin chloride. The water solution after standing will give a green colour with alkali owing to admixture with the yellow salt of the isomer

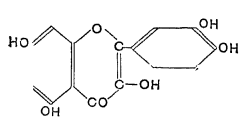
(*b*) Add alkali A deep blue colour is produced A crude extract of the fresh petals made as in Expt 101 will give a green or bluish-green colour with alkali owing to the presence of the accompanying flavone.

(*c*) Add amyl alcohol and sulphuric acid No colour is taken up by alcohol The pigment is present as the glucoside peonin Boil another portion with sulphuric acid and add amyl alcohol The pigment is partly hydrolyzed and the peonidin goes into solution in the alcohol

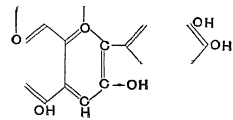
(*d*) Reduce another portion with zinc dust and hydrochloric acid The colour returns after filtering

In considering the anthocyan pigments, the question now arises—What is the chemical significance of the various shades in the living plant? Apparently the same pigment may be present in two flowers of totally different colours, as in the blue Cornflower and the magenta *Rosa gallica* It has been suggested that in such cases the pigment is modified by other substances present in the cell-sap thus it may be present in one flower as a potassium salt, in another as an oxonium salt of an organic acid, and in a third in the unaltered condition But exactly how these conditions are brought about is not clear. In one or two cases, moreover, where there is a red or pink variety of a blue or purple flower, the variety, when examined, has been found to contain a different pigment and one less highly oxidized than that in the species itself The above phenomena are exemplified in the Cornflower (*Centaurea Cyanus*) The flowers of the blue type contain the potassium salt of cyanin, the purple variety, cyanin itself, while those of the pink variety contain pelargonin

The mode of origin of anthocyan pigments in the plant is as yet obscure It has been suggested (Wheldale, 29) that they have an intimate connexion with the flavone and flavonol pigments, which can be seen at once by comparing the structural formula of quercetin with that suggested for cyanidin



Quercetin



Cyanidin

All the anthocyan pigments so far isolated, however, have been found to contain the flavonol, and not the flavone, nucleus

Just as in the case of the flavone and flavonol pigments, some of the anthocyan pigments are specific, while others, on the contrary, are common to various genera and species. Also more than one anthocyan pigment may be present in the same plant.

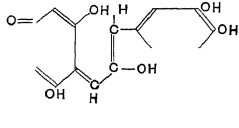
It will be pointed out later that small amounts of a substance identical with cyanidin are said to be formed by reduction of quercetin with nascent hydrogen, but this does not necessarily prove that the formation of anthocyan pigments in the plant takes place on the same lines. If we compare the formulae for a number of anthocyan with flavone and flavonol pigments, it is seen that they may be respectively arranged in a series, each member of which differs from the next by the addition of an atom of oxygen

Luteolin, kaempferol and fisetin $C_{15}H_{10}O_6$	Pelargonidin $C_{15}H_{10}O_5$
Quercetin $C_{15}H_{10}O_7$	Cyanidin $C_{15}H_{10}O_6$
Myricetin $C_{15}H_{10}O_8$	Delphinidin $C_{15}H_{10}O_7$

The relationship between these two classes of substances in the plant can only be ascertained by discovering which flavone, flavonol and anthocyan pigments are present together, and then to determine whether the relationship is one of oxidation or reduction, a problem which has not yet received adequate attention (Everest, 6)

A reaction which is of interest in connexion with the relationship between the above two classes of pigments is that which takes place when solutions of some flavone or flavonol pigments are treated with nascent hydrogen If an acid alcoholic solution of quercetin is treated with zinc dust, magnesium ribbon or sodium amalgam, a brilliant magenta or crimson solution is produced, and this solution gives a green colour with alkalies (Combes, 5) The red substance thus produced has been termed "artificial anthocyanin" or allocyanidin The product is not a

true anthocyan pigment but has, it is suggested, an open formation (Willstatter, 36):



It is said, however, to contain small quantities of a substance identical with natural cyanidin from the Cornflower (Willstatter, 36). The fact that small quantities of a natural anthocyan pigment can be obtained artificially from a hydroxyflavonol by reduction does not necessarily imply that one class is derived from the other in the living plant

From the above reaction of quercetin the result follows that when many plant extracts [most plants (see p 94) contain flavone or flavonol pigments] are treated with nascent hydrogen, artificial anthocyan pigment is produced Moreover, it seems probable that if the yellow pigments acted upon are in the glucosidal state, and if the reduction takes place in the cold, allocyanin (the glucoside of allocyanidin) is formed and the product is not extracted from solution by amyl alcohol But if the flavone is non-glucosidal, or if the solution is boiled before or after reduction, then allocyanidin (non-glucosidal) is formed and is extracted by amyl alcohol

Expt 109 *Formation of allocyanidin from quercetin* Make an alcoholic solution of a little of the glucoside of quercetin prepared from either *Narcissus* or *Cheiranthus* (see Expts 98 and 100) Acidify with a little strong hydrochloric acid and heat on a water-bath Add a little zinc dust from time to time. A brilliant magenta colour due to allocyanidin is produced To a little of this solution add some alkali: a green colour is produced If the alcohol and hydrochloric acid are evaporated off, and a little water and sulphuric acid added, on shaking up with amyl alcohol, all the allocyanidin passes into the amyl alcohol (The distribution of the allocyanidin in the amyl alcohol is greater with aqueous sulphuric acid than with aqueous hydrochloric acid)

Expt 110 *Formation of allocyanin from quercetin.* Make a suspension of the glucoside of quercetin from *Cheiranthus* or *Narcissus* (see Expts 98 and 100) in about 2N sulphuric acid, and then add zinc dust (or a drop of mercury about the size of a pea and a little magnesium powder) in the cold The red or magenta colour is gradually developed. Divide the coloured solution into two parts in two test-tubes. Boil one for 5–10 minutes Then add amyl alcohol to each In the unboiled test-tube the amyl alcohol extracts no colour, since allocyanin is present. In the boiled test-tube allocyanidin is taken up by the amyl alcohol as in Expt. 109

Expt 111 *Formation of allocyanin and allocyanidin from plant extracts* For this purpose the yellow varieties "Primrose" or "Cloth of Gold" of the Wallflower (*Cheiranthus Cheiri*) can be used The flowers are pounded in a mortar, extracted with cold water, the water extract acidified with sulphuric acid, and zinc dust (or mercury and magnesium powder as above) added A red coloration is slowly developed To some of the red solution add amyl alcohol The colour is not abstracted (allocyanin) Boil another portion The allocyanin is thus converted into allocyanidin which is then taken up on addition of amyl alcohol

The Oxidizing Enzymes

There is a certain group of enzymes of which we have most information in their connexion with aromatic substances. These are the oxidizing enzymes

The presence of such enzymes in plants was long ago associated with the following phenomena If the expressed juices, or water extracts of the tissues, of some plants are added to a solution of guaiacum gum, in the presence of air, a deep blue colour is obtained in a short time On the other hand, expressed juices, or water extracts, of other plants added to guaiacum solution produce no blue colour On addition, however, of a few drops of hydrogen peroxide, in the latter case, the blue colour rapidly develops. Plants are said to contain an oxidase when the extracts give the blue colour with guaiacum tincture alone. Those, of which the extracts only bring about blueing on addition of hydrogen peroxide, are said to contain a peroxidase (Chodat, 1)

Expt 112 *Demonstration of the presence of an oxidase* Take a portion of a fresh Potato tuber and pound well in a mortar with a little water Filter, and to the extract, in a white evaporating dish, add a few drops of 1% guaiacum solution in alcohol The guaiacum tincture will be found to give a blue colour at once with the potato extract.

A control tube should be prepared by *well* boiling some of the water extract of the tuber, cooling and then adding guaiacum. No colour is produced, owing to the fact that the enzyme has been destroyed by boiling Other tissues which may be used to demonstrate the above reactions are: root of beet and fruits of apple, pear and plum

Guaiacum gum is obtained from two West Indian species of Guaiacum trees, *G officinale* and *G sanctum*, partly as a natural exudation and partly by means of incisions. It gives a yellow solution with alcohol which contains guaiaconic acid, and the latter, on oxidation, yields guaiacum blue As far as possible, inner portions of the resin lumps should be used, as the resin oxidizes in air, and then may give unreliable results It is best to make the tincture freshly before use, and, as a precaution, to boil it on a water-bath with a little animal charcoal and filter Guaiacum gum tends to form peroxides on exposure to air, and these are removed by the above treatment.

Expt 113 *Demonstration of the presence of a peroxidase* Repeat the above experiment, using scales from an Onion bulb Add a few drops of hydrogen peroxide solution Green pea tissue may also be used No blue colour is produced until after the addition of hydrogen peroxide

If a systematic examination is made of genera from various natural orders with the guaiacum test, it will be found that the oxidase reaction is shown by all, or nearly all, genera of some orders, for instance most Umbelliferae, Labiatae, Boraginaceae and many Compositae. In other orders, i e Cruciferae and Crassulaceae, the peroxidase reaction is predominant

Moreover, the oxidase-containing genera show another phenomenon, as follows If the tissue extracts are left in air, they become dark-coloured, generally brown or reddish-brown If the plants themselves are bruised or injured, they turn brown The same effect may be produced very rapidly by the action of chloroform vapour These phenomena, the darkening of extracts and the discoloration, both on injury and in chloroform, will be found to be absent from plants giving the peroxidase reaction only

Expt 114 *To show the distribution of oxidases and peroxidases in various plants, and the correlation between the presence of oxidase and browning on injury or in chloroform vapour* Take a selection of the plants given below, and in each case grind up a portion of the plant in a mortar with a little water and filter. Divide the filtrate into two parts in small porcelain dishes Allow one part to stand in air, and note the darkening in colour in cases where an oxidase is present. To the other add a few drops of guaiacum To extracts containing a peroxidase only, after 5–10 minutes, add in addition a few drops of hydrogen peroxide Further, small pieces of the plants to be tested should be placed in a corked flask containing a few drops of chloroform, and the development of browning noted in the case of plants containing an oxidase For demonstration of oxidases the following plants may be used. Christmas Rose (*Helleborus niger*), Dandelion (*Taraxacum officinale*), Forget-me-not (*Myosotis*), Hawthorn (*Crataegus*) and White Dead Nettle (*Lamium album*) For peroxidases *Arabis*, *Aubrietia*, Buttercup (*Ranunculus acris*), Pea (*Pisum sativum*), Stock (*Matthiola*), Wallflower (*Cheiranthus Cheiri*) and Violet (*Viola*).

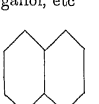
Various hypotheses have been suggested in explanation of the reactions of oxidizing enzymes The one most generally accepted (Chodat, 1) is that the oxidase consists of two components, a peroxide and a peroxidase. The peroxide may be either hydrogen peroxide or some organic peroxide The peroxidase acts upon the peroxide, depriving it of an atom of oxygen which is transferred, in an "active" condition, to some substance (acceptor) which becomes oxidized Guaiacum is the acceptor in the above experiments The reaction may be approximately expressed thus

$$AO + O_2 = AO_2 \text{ (peroxide)}.$$
$$AO_2 + \text{peroxidase} = AO + O$$

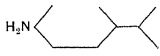
The above conception would represent a system which is capable of absorbing molecular oxygen and of transforming it into active oxygen. In this way substances can be oxidized which would not be affected by molecular oxygen to any extent

Accordingly, on the basis of the above hypothesis, it is assumed that an organic compound which can act as a peroxide is present in the expressed plant juices or extracts which give the oxidase reaction When this organic peroxide is absent, hydrogen peroxide must be added to complete the oxidase system

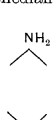
The above definition of oxidases and peroxidases is based upon their behaviour towards guaiacum. There are, however, a number of other oxidizable substances, such as α-naphthol, benzidine, *p*-phenylenediamine, pyrogallol, etc



α-Naphthol



Benzidine



p-Phenylenediamine

which are only oxidized by oxidases with the assistance of hydrogen peroxide, the oxidase system being insufficient, they are also of course oxidized by the hydrogen-peroxide-peroxidase system Such substances (in the presence of hydrogen peroxide) are used as tests for oxidizing enzymes.

Expt 115 *Additional tests for oxidizing enzymes* To a few c.c. of an extract of Potato tuber (Expt. 112) in a small evaporating dish, add a few drops of the following reagents, and subsequently a few drops of hydrogen peroxide

(*a*) A 1 % solution of α-naphthol in 50 % alcohol. A blue colour is developed.

(*b*) A 1 % solution of benzidine in 50 % alcohol A blue colour is developed

(*c*) A 1 % solution of *p*-phenylenediamine hydrochloride in water A greenish colour is developed

The peroxidases, like other enzymes, can be extracted either with water or dilute alcohol and precipitated from solution by strong alcohol

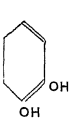
Expt 116. *Preparation of peroxidase from Horse-radish* (Cochlearia) *roots* Mince up the Horse-radish roots in a mincing machine The product is allowed to stand for 24 hrs. to enable the glucoside, potassium myronate, to be hydrolyzed by the enzyme, myrosin Then extract with 80 % alcohol The alcohol is decanted off, and the residue pressed free from alcohol in a press. The residue is next extracted with 40 % alcohol for 48 hrs, filtered and precipitated with 90 % alcohol The precipitate, which contains the peroxidase, is filtered off Dissolve up in water and make the test for peroxidases (Expt 113).

The oxidation of pyrogallol, in the presence of a peroxidase and hydrogen peroxide, has been used as a method for estimating the activity of these enzymes. Solutions of known strength of pyrogallol and hydrogen peroxide are used, and to the mixture a solution of a known weight of prepared peroxidase is added An oxidation product, termed purpurogallin, is formed After a definite time, the reaction is stopped by adding acid, and the purpurogallin extracted by ether The ether extract is colorimetrically compared with an extract containing a known amount of purpurogallin (Willstatter and Stoll, 47).

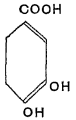
Expt 117 *Outline of method for estimating peroxidase by formation of purpurogallin* Make a solution of 0·5 gm of pyrogallol in 200 c c of distilled water, and add to it 1 c c of 5 % hydrogen peroxide Then add about 5 c c of a solution of Horse-radish peroxidase from Expt 116 After 5 minutes add to half the mixture 25 c c of dilute sulphuric acid and extract the purpurogallin with ether in a separating funnel The purpurogallin will be extracted by the ether, giving a yellow solution Allow the other half of the mixture to stand The colour will deepen, and a reddish deposit of purpurogallin will be precipitated Examine a little of the deposit under the microscope It will be found to consist of sheaves of crystals

A solution of peroxidase from *Alyssum* leaves [Expt 119 (*b*)] can also be used

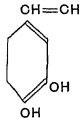
It has recently been shown (Wheldale Onslow, 11) that the peroxidase element (Expt. 118) of certain oxidases (fruit of Pear, tuber of Potato) can directly oxidize the phenol catechol (see p. 88), as well as other substances containing the catechol grouping, that is two hydroxyl groups in the ortho position, e g. protocatechuic acid and caffeic acid



Catechol



Protocatechuic acid



Caffeic acid

A brown colour is produced, giving rise to some oxidation product which will then act as a peroxide, the peroxidase being present, to form the system (oxidase) which will blue guaiacum.

It appears probable that the above phenomenon, as illustrated in the Pear and Potato, affords an explanation of the reaction of the oxidizing enzymes of all plants which give the oxidase reaction and turn brown on injury. For such plants usually contain an aromatic substance with the catechol grouping. On the death of the cell (which may be brought about, either by injury, or treatment with chloroform vapour, i e on autolysis, see p. 19) this aromatic substance is oxidized by the

peroxidase with the formation of a brown colour (browning on injury) and an oxidation product which then acts as an organic peroxide (as postulated above) in the oxidase system

Further, the peroxidases, of plants which give the oxidase reaction and brown on injury, appear to be specific in their action on the catechol grouping and will not oxidize other phenolic groupings, i e those of resorcinol, hydroquinone, pyrogallol, etc

In plants (e g *Alyssum*) which only give the peroxidase reaction and do not brown on injury, the above-mentioned aromatic compounds are not present, nor do the peroxidases of such plants act on catechol

It should, however, be borne in mind that in some plants the components of the oxidase system may be present, but the reaction is masked by the presence of an excess of tannin, sugars, etc

Hence it appears that the oxidase reaction of plants (as detected by guaiacum) is the outcome of post-mortem changes after the death of the cell. It is possible, however, that the processes giving rise to it may take place to some extent, though under control, in the living cell.

It has also been shown that in the Pear fruit and Potato tuber (Wheldale Onslow, 11) it is possible by chemical methods to separate the elements which go to make up the oxidase system, i e the peroxidase and the aromatic substance If the tissues are ground up rapidly with alcohol, and filtered before oxidation can take place, and the process repeated, the aromatic substance, which is soluble in alcohol, is washed away, and a water extract of the residue gives only a peroxidase reaction. From the alcoholic extract the aromatic substance can be precipitated as a lead salt, the lead then removed as insoluble lead sulphate, and the aromatic set free again in solution. If the peroxidase is then added to the solution of the aromatic, the brown colour appears together with the formation of the oxidation product (peroxide), and the mixture will give the blue reaction of an oxidase with guaiacum.

Expt 118 *Resolution of the components of the oxidase in the Potato tuber* (A) *Separation of peroxidase* Cut a few thin slices from a peeled potato and put them in a mortar which contains sufficient 96 % alcohol to prevent, as far as possible, exposure to the air, and pound them thoroughly Filter quickly on a filter-pump, and repeat the process several times until a colourless powder, consisting of cell-residues, starch, etc. is obtained The enzymes (including the peroxidase) of the cells are precipitated by the alcohol and remain in the cell-residue Make a water extract of the white powder and filter To a portion of the filtrate add a few drops of guaiacum tincture . no blue colour is given Add further a few drops of dilute hydrogen peroxide . a blue colour appears (B) *Separation of the aromatic substance.* Take about 500 gms of freshly peeled potato tuber, cut it into thin slices and drop

them as rapidly as possible into a flask containing 250 c c of boiling 96 % alcohol on a water-bath Continue boiling for 15 mins, and then filter Evaporate off the alcohol from the filtrate, take up the residue in a little water, warm and filter To the filtrate add concentrated lead acetate solution until a precipitate ceases to be formed Filter off the precipitate, which is pale yellow in colour, stir up in a little water and add 10 % sulphuric acid drop by drop until the yellow colour is destroyed, and the lead is converted into lead sulphate Filter off the lead sulphate the filtrate contains the aromatic substance in solution Neutralize the solution carefully with 1 % caustic soda and make the following tests with separate portions in small evaporating dishes

(*a*) Add a drop of ferric chloride solution a deep green colour appears Add further a few drops of 1 % sodium carbonate solution The green colour changes to a bluish- and finally, a reddish-purple This reaction is characteristic of aromatic compounds containing the catechol grouping, i e two hydroxyl groups in the ortho position (see p 110)

(*b*) Add a little of the peroxidase solution prepared in (A) The mixture will gradually turn brown owing to the oxidation of the aromatic by the peroxidase

(*c*) To (*b*) add a few drops of guaiacum tincture A blue colour is given owing to the presence of the peroxide formed in (*b*), the oxidase system being now complete

Expt 119 *Action of peroxidases on catechol.* (*a*) *The peroxidase of the Potato tuber* (*or Pear fruit*) Make a 1 % solution of catechol in distilled water To some of this solution, in a small evaporating dish, add a little of the peroxidase solution from Expt 118 (A) Note that the catechol solution gradually turns brown Add further a few drops of guaiacum tincture A blue colour appears (*b*) *Peroxidase of* Alyssum *leaves* Pound up 2–3 *Alyssum* leaves in a mortar with some 96 % alcohol, and filter on a filter-pump Repeat the process until the residue is practically colourless Extract the residue with a little distilled water and filter Proceed as in (*a*) No browning of catechol takes place and no blue colour is formed on the subsequent addition of guaiacum

For section (*a*) the following material may also be used fruits of Apple and Greengage, flowers of Horse Chestnut (*Aesculus*) and leaves of Pear, the method of preparation in (*b*) being employed For section (*b*) flowers of white *Arabis* may also be used.

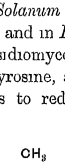
If in the preparation of the peroxidase from the Potato tuber, the tissue is allowed to brown before extracting with alcohol, the cell-residue is tinged with brown and, on extraction with water, the filtrate will give an oxidase reaction with guaiacum. This is to be explained by the fact that the peroxide has been adsorbed by the tissue residue. This phenomenon is probably the explanation of the preparation of some oxidases called "laccases" Such enzymes have been obtained by the precipitation with strong alcohol of the expressed juices (containing peroxide since they were obtained by crushing the tissues) of plants which brown on injury The enzyme and other organic matter is precipitated and carries with it the peroxide.

The browning of plants on injury, as already mentioned, is very wide-spread: more rarely, the same type of reaction may lead to the formation of variously coloured products, other aromatic substances in addition to the peroxide being in all probability involved. The following examples may be quoted. In some of the Higher Fungi, *Boletus luridus* and *B. cyanescens*, when the pileus is broken across or injured, the tissue rapidly turns a deep Prussian blue on exposure to the air. Also in the Fungus, *Russula nigricans*, the colour changes from pink or reddish to black. The latex of the tree, *Rhus vernicifera*, when exposed to air, turns black and constitutes the product, Japanese lacquer.

Analogous pigments formed after death have been observed in *Schenckia blumenaviana* (Rubiaceae) where the whole plant turns bright red in chloroform vapour, as well as in species of *Jacobinia* and *Aloe*. Blue pigments are also formed after death in the Teasel (*Dipsacus*), in the flowers of an Orchid (*Phajus*), in the Dog's Mercury (*Mercurialis perennis*), annual Mercury (*M. annua*) and in the well-known indigo-producing plants, the Woad (*Isatis tinctoria*) and the Indigo (*Indigofera*) (Walther, 28) (see p. 152). It has not been shown, however, that oxidizing enzymes are present in all the above cases.

There is a well-known oxidizing enzyme, tyrosinase, which is somewhat specific in its action in that it will only oxidize tyrosine and a few other substances, such as *p*-cresol. It occurs in the tubers of the Dahlia (*Dahlia variabilis*) and Potato (*Solanum tuberosum*), in grains of barley (*Hordeum*) and wheat (*Triticum*) and in *Papaver orientale*. In the lower plants it is found among the Basidiomycetes.

When tyrosinase acts upon tyrosine, a yellowish-pink colour is first produced which rapidly darkens to red, brown and finally black. A solution of *p*-cresol



can be used as a delicate test for the enzyme. If the enzyme is present, a yellowish- or orange-red colour is formed.

Tyrosinase of the Potato tuber can be precipitated from a water extract with absolute alcohol: or if the potato tissue is extracted with cold 96% alcohol, the enzyme is precipitated and remains in the

tissue residue, as does the peroxidase [Expt 118 (A)], but the tyrosine is almost entirely washed away

Expt 120 *Demonstration of the presence of tyrosinase in the Potato* Take about half a potato and proceed as in the preparation of peroxidase [see Expt 118 (A)] Roughly dry the powder left on the filter and then add about 100 c c of water and allow to stand for 15 mins Filter, and divide the filtrate into six portions *a*, *b*, *c*, *d*, *e* and *f* Make a suspension of a little tyrosine in water (tyrosine is only slightly soluble in cold water)

To *a* add 5 c c of tyrosine suspension

To *b* add 5 c c of tyrosine suspension and a few drops of hydrogen peroxide

To *c* add 5 c c of tyrosine suspension and boil

To *d* add a few drops of hydrogen peroxide only.

To *e* add some *p*-cresol

To *f* nothing is added

Plug all the tubes with cotton-wool, put in an incubator at 38° C for 2–3 hrs. Note that tubes *a* and *b* fairly rapidly turn red, then brown and finally black Tubes *d* and *f* may darken a little owing to the action of tyrosinase and peroxidase on the traces of plant aromatics left in the tissue Tube *c* remains unaltered Tube *e* gives an orange-red colour

Another point which may be touched upon is the significance of the oxidizing enzymes in plant metabolism The facts as they present themselves may be stated thus On death or injury we discover a system (oxidase) in some plants which can oxidize certain acceptors, either artificially introduced, such as guaiacum, or other aromatic substances present in the plant itself.

The question is whether the oxidase system as described above functions in metabolism for the purpose of oxidation and respiration in the living plant. If it does, there are various suggestions as to how it may act For instance, it is possible that aromatic substances are oxidized to pigments by the oxidase system, and the pigment is then deoxidized by products of anaerobic respiration, hence constituting a mechanism for this function Such pigments have been termed "respiration pigments" (Palladin, 12–16). It is suggested that they are not present in any quantity in the living plant and their production is controlled by oxidizing and reducing systems:

autoxidizable substance + molecular oxygen = peroxide
peroxide + peroxidase = active oxygen
aromatic substance (acceptor or chromogen) + active oxygen = pigment
pigment + reducing substance = chromogen.

The above hypothesis is only applicable to plants containing oxi-

dases, so that some modification would be necessary to fit the case of plants containing peroxidases only

It should be mentioned that the aromatic substances which become oxidized are frequently present in the plant as glucosides, that is, some of the hydroxyl groups are replaced by sugar Indoxyl, the precursor of indigo, forms a good example, as it is present in the plant as a colourless glucoside, indican (see p. 152). It is probably only after the hydrolysis of the glucosides that the aromatic substances can become oxidized.

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CHAPTER VIII

THE PROTEINS AND PROTEASES

No class of compounds is of more fundamental significance than the proteins The matrix of protoplasm largely consists of proteins in the colloidal state, and, without doubt, they occur to some extent in the same condition in the cell-sap. They are also found in the cell in the solid state, in the form of either amorphous granules, termed aleurone, or crystalline or semi-crystalline bodies, termed crystalloids. Both solid forms constitute "reserve material" and are often found in seeds, tubers, bulbs, buds and roots.

Plant proteins may be classified on the following plan.

1 The simple proteins.
   (*a*) Albumins.
   (*b*) Globulins
   (*c*) Prolamins (Gliadins).
   (*d*) Glutelins
2 Conjugated proteins
   (*a*) Nucleoproteins
3. Derived proteins
   (*a*) Metaproteins
   (*b*) Proteoses (Albumoses)
   (*c*) Peptones
   (*d*) Polypeptides

Although they are present in every cell in all parts of plants, little, however, is known of plant proteins, except of those in seeds, because of the difficulties of obtaining them in sufficiently large quantities, and of separating them from each other

Proteins are in the colloidal state when in so-called solution, and are unable to diffuse through parchment membranes The proteoses and peptones, however, which have simpler molecules, can diffuse through such membranes

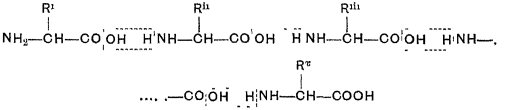
The vegetable proteins are soluble in various solvents according to the nature of the protein; some are soluble in water, others in dilute salt solutions, others, again, in dilute alkalies, and a few in dilute alcohol. Vegetable albumins are coagulated from solution on boiling,

but most of the globulins, unlike the corresponding animal products, are only imperfectly coagulated on heating and some not at all. The precipitate formed when coagulation is complete will not go into solution again either in water, acid, alkali or salts Alcohol precipitates the proteins, in the case of animal proteins, the precipitate becomes coagulated and insoluble if allowed to remain in contact with the alcohol, but this does not appear to be so with plant proteins

In addition, certain neutral salts, the chlorides and sulphates of sodium, magnesium and ammonium, have the property of precipitating proteins (except peptones) from solution when added in sufficient quantity. The protein is quite unchanged in precipitation and can be made to go into solution again The various proteins are precipitated by different concentrations of these salt solutions (see p 124)

The salts of calcium and barium and the heavy metals produce insoluble precipitates with the proteins, and in this case the reaction is irreversible

In regard to chemical composition, the proteins contain the elements carbon, hydrogen, nitrogen, oxygen and sulphur. There is every reason to believe that the protein molecule is constituted of amino-acids condensed, with elimination of water, on the plan which may be depicted as follows



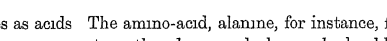
Conversely, when the proteins are acted upon by hydrolyzing enzymes, a series of hydrolytic products are formed which have smaller molecules than the original proteins. They may be enumerated as

1 Albumoses
2 Peptones
3 Amino-acids

In the same way when proteins are boiled with acids, a number of the amino-acids are obtained as an end-product

The above amino-acids may be either aliphatic or aromatic, and they are characterized by having one or more hydrogen atoms, other than those in the carboxyl groups, replaced by the group $-NH_2$. Thus they are acids by virtue of the carboxyl groups, and bases by virtue of the $-NH_2$ groups towards strong acids they act as bases, and towards

strong bases as acids The amino-acid, alanine, for instance, forms salts, sodium amino-propionate with a base, and alanine hydrochloride with an acid ·



Substances behaving in this way have been termed "amphoteric" electrolytes (see also p 15)

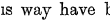
In the proteins, which are formed by condensation, as explained above, there are always some NH_2 and COOH groups left uncombined Hence a protein must, in the same way, have the properties of both an acid and a base

The amino-acids which are obtained by the hydrolysis of plant proteins may be classified as follows

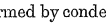
Aliphatic compounds

Mono-carboxylic mono-amino acids

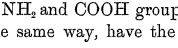
Glycine or α-amino-acetic acid



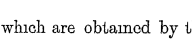
Alanine or α-amino-propionic acid



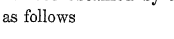
Valine or α-amino-iso-valeric acid



Leucine or α-amino-iso-caproic acid



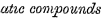
Iso-leucine or α-amino-β-methyl-β-ethyl-propionic acid



Serine or α-amino-β-hydroxy-propionic acid

Dicarboxylic mono-amino acids

Aspartic acid or α-amino-succinic acid

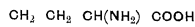


Glutamimic acid or α-amino-glutaric acid

Mono-carboxylic di-amino acids

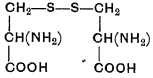
Arginine or δ-guanidine-α-amino-valeric acid

Lysine or α-ε-di-amino-caproic acid



Dicarboxylic di-amino acid

Cystine (dicysteine) or di-β-thio-α-amino-propionic acid

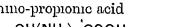


Aromatic compounds.

Mono-carboxylic mono-amino acids

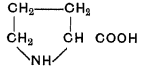
Phenyl-alanine or β-phenyl-α-amino-propionic acid

Tyrosine or *p*-hydroxy-phenyl-alanine

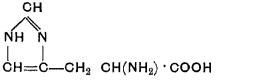


Heterocyclic compounds.

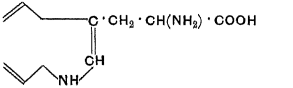
Proline or α-pyrrolidine-carboxylic acid



Histidine or β-imidazole-alanine



Tryptophane or β-indole-alanine $C_8H_6N \cdot CH_2 \cdot CH(NH_2) \cdot COOH$



Different proteins are formed by various combinations of the above acids and hence give different amounts on hydrolysis.

There are certain properties and chemical reactions by means of

which proteins can be detected These are illustrated in the following experiment

Expt 121 *Tests for proteins* Weigh out about 10 gms of dried peas (*Pisum*), grind them in a coffee-mill and then add 100 c c of water to the ground mass Allow the mixture to stand for an hour Filter, and make the following tests with the filtrate (see p 133)

(*a*) *The xanthoproteic reaction* To a few c c of the protein solution in a test-tube add about one-third of its volume of strong nitric acid A white precipitate is formed (except in the case of proteoses, peptones, etc) On boiling, the precipitate turns yellow, and may partly dissolve to give a yellow solution Cool under the tap, and add strong ammonia till the reaction is alkaline The yellow colour becomes orange The precipitate is due to the fact that metaprotein (see p 127) is formed by the action of acid on albumins or globulins, and this metaprotein is insoluble in strong acids The yellow colour is the result of the formation of a nitro-compound of some aromatic component of the protein, such as tyrosine, tryptophane and phenylalanine

(*b*) *Millon's reaction* To a few c c of the protein solution add about half its volume of Millon's reagent.[1] A white precipitate is formed On warming, the precipitate turns brick-red, or disappears and gives a red solution The white precipitate is due to the action of the mercuric nitrate on the proteins The reaction is characteristic of all aromatic substances which contain a hydroxyl group attached to the benzene ring The aromatic complex in the protein to which the reaction is due is tyrosine

(*c*) *The glyoxylic reaction (Hopkins and Cole)* To about 2 c c of protein solution add an equal amount of "reduced oxalic acid"[2] Mix the solutions, and then add an equal volume of concentrated sulphuric acid, pouring it down the side of the tube A purple ring forms at the junction of the two liquids If the liquids are mixed by shaking the tube gently, the purple colour will spread throughout the solution. The substance in the protein molecule to which the reaction is due is tryptophane If carbohydrates are present in the liquid to be tested, the colour is not good, owing to blackening produced by the charring with the strong sulphuric acid.

(*d*) *The biuret reaction* To a few c c of the protein solution add an excess of sodium hydrate and a drop of a 1 % solution of copper sulphate A violet or pink colour is produced The reaction is given by nearly all substances containing two CONH groups attached to one another, to the same nitrogen atom, or to the same carbon atom The cause of the reaction with proteins is the presence of one or more

[1] This reagent is made by dissolving 30 c.c of mercury in 570 c c of concentrated nitric acid and then adding twice its bulk of water It contains mercurous and mercuric nitrates, together with excess of nitric acid and a little nitrous acid.

[2] Reduced oxalic acid is prepared as follows (*a*) Treat 500 c c of a saturated solution of oxalic acid with 40 gms of 2 % sodium amalgam When hydrogen ceases to be evolved, the solution is filtered and diluted with twice its volume of distilled water. The solution contains oxalic acid, sodium binoxalate and glyoxylic acid (COOH · CHO). (*b*) Put 10 gms of powdered magnesium into a flask and just cover with distilled water. Add slowly 250 c c of saturated oxalic acid, cooling under the tap Filter off the insoluble magnesium oxalate, acidify with acetic acid and dilute to a litre with distilled water

groupings formed by the condensation of the carboxylic group of an amino-acid with the amino group of another amino-acid (see p. 119)

(*e*) *The sulphur reaction* Boil a few c c of the protein solution with some 40 % sodium hydrate for two minutes, and then add a drop or two of lead acetate The solution turns black This reaction is due to the formation of sodium sulphide by the action of the strong alkali on the sulphur of the protein On addition of the lead salt, a black precipitate of lead sulphide is formed The sulphur in the protein molecule is mainly present as cystine

For the following reactions, a protein solution free from other impurities is required For this purpose take 40 gms of ground peas, add to the meal about 200 c c 10 % sodium chloride solution, and allow the mixture to stand, with occasional stirring, for 3–12 hrs (see p. 134) Then filter off the extract, first through muslin, and, subsequently, through filter-paper Put the extract to dialyze for 24 hrs in a collodion dialyzer[1] until the protein is well precipitated (Toluol should be added to the liquid in the dialyzer) Then filter off the protein. Reserve half, and dissolve the other half in about 50 c c of 5 % sodium nitrate solution With this solution (after reserving a portion for Expt 123) make the following tests

(*f*) *Precipitation by alcohol* To a few c c in a test-tube, add excess of absolute alcohol The protein is precipitated

(*g*) *Precipitation by the heavy metals.* Measure out a few c.c of the protein solution into three test-tubes, and add respectively a little (1) copper sulphate solution, (2) lead acetate solution, (3) mercuric chloride solution the protein is precipitated in each case

The following test cannot be demonstrated on the Pea protein, since carbohydrates are absent in this case It can, however, be demonstrated in later experiments (see p 130)

(*h*) *Molisch's reaction* To a few c c of the protein solution add a few drops of a 1 % solution of α-naphthol in alcohol. Mix, and then run in an equal volume of strong sulphuric acid down the side of the tube A violet ring is formed at the junction of the two liquids The reaction signifies the existence in the protein of a carbohydrate group which gives rise, on treatment with acid, to furfural The latter then condenses with α-naphthol to give a purple colour (see also Expts 38, 44, 46)

(*i*) *Precipitation by salts of alkaline earth metals* To a few c c of the protein solution add a little barium chloride solution A precipitate is formed on standing

(*j*) *Precipitation by neutral salts* Saturate a few c c of the protein solution with finely powdered ammonium sulphate. The protein is precipitated or "salted out"

Since from a neutral salt solution the pea globulin is precipitated by acid (see p 125), the tests (*k*)–(*m*) should be carried out with a solution of the protein in dilute acid. Dissolve, therefore, the remainder of the solid pea globulin in about 40 c c of 10 % acetic acid, filter, and make the following tests:

(*k*) *Precipitation by tannic acid.* Add a little tannic acid solution: the protein is precipitated

[1] The collodion solution is made by adding 75 c c. of ether to 3 gms of well-dried pyroxylin, allowing it to stand for 10–15 minutes and then adding 25 c c of absolute alcohol The dialyzers are prepared by coating the inside of a large test-tube with the solution and then filling with water, after the film is sufficiently dried so as not to be wrinkled by touching with the finger. The sac can then be withdrawn from the tube

(*l*) *Precipitation by Esbach's solution*[1] Add a little Esbach's solution the protein is precipitated

(*m*) *Precipitation by phosphotungstic acid* Add a little 2 % solution of phosphotungstic acid in 5 % sulphuric acid the protein is precipitated

The substances used in the tests (*k*)–(*m*) are termed "alkaloidal reagents" because they also cause precipitation of alkaloids (see Chap X).

We are now in a position to deal with the different groups of proteins in detail

Albumins. Very few vegetable albumins have been investigated. They can be best defined as proteins which are *soluble in water* and are coagulated by heat Animal albumins are distinguished by the fact that they are not precipitated by saturating their neutral solutions with sodium chloride or magnesium sulphate, nor are they precipitated by half-saturation with ammonium sulphate This distinction cannot be applied to vegetable proteins, since some are precipitated by the above treatment It is often not easy to determine whether a plant protein is an albumin, on account of the difficulty of removing traces of salts acids or bases which cause solubility, and also of separating the albumins from the globulins with which they occur Albumins are however probably widely distributed in plant tissues.

The best-known albumins are:

Leucosin, which occurs in the seeds of Wheat (*Triticum vulgare*), Rye (*Secale cereale*) and Barley (*Hordeum vulgare*)

Legumelin, which occurs in seeds of the Pea (*Pisum sativum*), Broad Bean (*Vicia Faba*), Vetch (*Vicia sativa*), Lentil (*Ervum Lens*) and some other Leguminous seeds

Phaselin, which occurs in the Kidney-bean (*Phaseolus vulgaris*).

Ricin, which occurs in the Castor-oil Bean (*Ricinus communis*).

Expt 122 *Demonstration of the presence of an albumin (leucosin) in wheat or barley flour (see also Expts* 128 *and* 130) Weigh out 10 gms. of wheat or barley flour, add 100 c c of distilled water and allow to stand, with occasional stirring, for 2–6 hrs. Then filter off the solution Slowly heat the solution to boiling, and note that a precipitate of coagulated protein is formed

Globulins. These may be defined as the proteins which are insoluble in water but soluble in dilute salt solutions, the concentration of the salt solution necessary for complete solution (see p. 125) varying with the salt or protein under consideration. It should be noted that, in making *water*-extracts of plant tissues, it may happen that globulins

[1] Esbach's solution is prepared by dissolving 10 gms. of picric acid and 10 gms. of citric acid in water and making the solution up to a litre

pass into solution to some extent owing to the presence of inorganic salts in the tissues themselves This has also already been illustrated in Expt 121 in which an extract of the globulin of the Pea was obtained by treating ground Pea seeds with distilled water only

It is characteristic of animal globulins that they are precipitated by saturation of their solutions with magnesium sulphate Many of the vegetable globulins cannot be precipitated by the above means, though they are all, as far as tested, precipitated by sodium sulphate at 33° C Many also (like animal globulins) are precipitated by half-saturation with ammonium sulphate, though others are not precipitated until their solutions are nearly saturated with this salt [see Expt. 121 (*j*)]

Unlike animal globulins, vegetable globulins are, as a rule, only imperfectly coagulated by heat, even on boiling.

Expt 123 *Demonstration of the coagulation of globulin* Heat a few c c of the solution of dialyzed Pea globulin (from Expt 121) in a test-tube Note that the protein is largely precipitated, but the solution does not become quite clear

One very important characteristic of the vegetable globulins is the ease with which a number of them can be obtained in crystalline form This result may be achieved by dialyzing a salt solution of the globulin. The salt passes out through the membrane, and the protein is deposited in the form of crystals An alternative method is to dilute the saline solution of globulin with water at 50–80° C until a slight turbidity appears Then warm further until this goes into solution, and cool gradually, when the protein will separate in crystals. The globulin, edestin, from seeds of the Hemp (*Cannabis sativa*) crystallizes very readily (see Expt. 133) and crystals can also be obtained of the globulins from the seeds of the Brazil nut (*Bertholletia excelsa*) (see Expt 136), the Flax or Linseed (*Linum usitatissimum*) (see Expt. 135), the Oat (*Avena sativa*) and the Castor-oil plant (*Ricinus communis*) (see Expt. 134), other globulins separate out on dialysis as spheroids, sometimes mixed with crystals

The solubilities of plant globulins are further complicated by the fact that some of these substances form acid salts which have different solubilities from the proteins themselves. Thus edestin is insoluble in water, but soluble in either dilute salt solution or acid. In the presence of acid it forms salts which are insoluble in dilute salt solutions Thus edestin in dilute acid solution is precipitated by a trace of salt, or in dilute salt solution by a trace of acid (see Expt 124) Legumin, on the other hand, from the Pea and other Leguminosae is soluble in water in

the free state combined with a small amount of acid as a salt, it is insoluble in water but soluble in neutral salt solution, that is, it has the solubilities of a globulin (see p 134)

Expt 124 *The formation of salts by edestin* Grind up 5 gms of seeds of the Hemp (*Cannabis sativa*) in a coffee-mill. Extract with 50 c c of warm (not above 60° C) 10% sodium chloride solution and filter Add a drop of strong hydrochloric acid to the filtrate Edestin chloride, which is insoluble in salt solutions, is precipitated Filter and drain off all the liquid, and then suspend the precipitate in distilled water Add 1 or 2 drops of hydrochloric acid carefully and stir till most or all of the precipitate goes into solution Filter, and to the filtrate add a few drops of saturated sodium chloride solution The edestin acid salt is again precipitated

The following is a list of the principal known globulins (Osborne, 2)

Legumin, in seeds of	Pea (*Pisum sativum*) Broad Bean (*Vicia Faba*). Vetch (*Vicia sativa*). Lentil (*Ervum Lens*).
Vignin, in seeds of	Cow Pea (*Vigna sinensis*).
Glycinin, in seeds of	Soy Bean (*Glycine hispida*)
Phaseolin (crystalline), in seeds of	Kidney Bean (*Phaseolus vulgaris*). Adzuki Bean (*P. radiatus*). Lima Bean (*P lunatus*).
Conglutin, in seeds of	Lupin (*Lupinus*)
Vicilin, in seeds of	Pea (*Pisum sativum*) Broad Bean (*Vicia Faba*) Lentil (*Ervum Lens*).
Corylin, in seeds of	Hazel Nut (*Corylus Avellana*).
Amandin, in seeds of	Almond (*Prunus Amygdalus*) Peach (*P. Persica*). Plum (*P. domestica*). Apricot (*P. Armeniaca*).
Juglansin, in seeds of...	European Walnut (*Juglans regia*) American Black Walnut (*J nigra*). American Butter-nut (*J cinerea*).
Excelsin (crystalline), in seeds of	Brazil Nut (*Bertholletia excelsa*).
Edestin, in seeds of	Hemp (*Cannabis sativa*).
Avenalin, in seeds of	Oat (*Avena sativa*).
Castanin, in seeds of	Sweet Chestnut (*Castanea vulgaris*).
Maysin, in seeds of	Maize (*Zea Mays*).
Tuberin, in tubers of	Potato (*Solanum tuberosum*).

Crystalline globulins have also been isolated from the following seeds but have as yet no distinctive names Flax (*Linum usitatissimum*), Squash (*Cucurbita maxima*), Castor-oil Bean (*Ricinus communis*), Coconut (*Cocos nucifera*), Cotton-seed (*Gossypium herbaceum*), Sunflower (*Helianthus annuus*), Radish (*Raphanus sativus*), Peanut (*Arachis hypogaea*), Rape (*Brassica campestris*) and Mustard (*Brassica alba*).

It will be seen that the majority of reserve proteins of seeds are globulins It is probable that native and artificial crystalline proteins are identical in many cases.

Prolamins These proteins are characterized by the fact that they are insoluble in water and dilute saline solutions, but are soluble in 70–90% alcohol. Such proteins are peculiar to plants, and are formed to a considerable extent in the seeds of cereals The principal ones which have been isolated are

Gliadin	found in the seeds	of	Wheat (*Triticum vulgare*)
„	„	„	Rye (*Secale cereale*)
Hordein	„	„	Barley (*Hordeum vulgare*)
Zein	„	„	Maize (*Zea Mays*).

The properties of the gliadins are demonstrated in Expts. 128, 129, 130 and 131.

Glutelins. The proteins of this group are insoluble in water, dilute saline solutions and in alcohol, but they are soluble in dilute alkalies. Glutenin of wheat is the only well-characterized member of this class which has so far been isolated, though other cereals most probably contain similar proteins. A protein of this nature has also been obtained from seeds of Rice (*Oryza sativa*). The properties of the glutelins are demonstrated in Expts. 128, 129 and 131.

Nucleoproteins. Though these proteins probably form constituents of all cells, the only members of the class investigated are those of the wheat embryo. This has been possible since nuclei form a large proportion of the tissue of the embryo They may be regarded as protein salts of nucleic acid, i.e. protein nucleates. On hydrolysis with acids or enzymes they split up into various proteins and nucleic acid. The nucleoproteins are also connected with the purins (see p. 164)

Metaproteins. These are hydrolytic products of albumins and globulins formed by the action of water or dilute acid or alkali. They are insoluble in water, strong mineral acids and all solutions of neutral salts, but are soluble in dilute acids and alkalies in the absence of any large amount of neutral salt.

Expt 125 *Reactions of metaprotein.* Dissolve about 1 gm of edestin (see Expt 133) in 50 c c of a 2% hydrochloric acid and keep on a boiling water-bath for 2 hrs. Neutralize with dilute sodium carbonate solution A copious precipitate of metaprotein separates out which is insoluble in water Filter off the precipitate and wash Make with it the following tests

(*a*) Dissolve up some of the precipitate again in 0 4 % hydrochloric acid To portions of the solution add (i) Dilute sodium carbonate the metaprotein is precipitated again and redissolves in excess. (ii) Concentrated hydrochloric acid the metaprotein is precipitated (iii) Boil some of the acid solution No coagulum is formed the metaprotein is not precipitated by boiling when in solution, and can still be precipitated by neutralizing with sodium carbonate

(*b*) Suspend some of the precipitate in water and boil Cool and add 0 4 % hydrochloric acid the precipitate is now insoluble, since the metaprotein is coagulated when boiled in suspension

(*c*) To some of the precipitate suspended in water, add gradually saturated ammonium sulphate solution the metaprotein is insoluble in all concentrations of the salt

Proteoses (albumoses) and peptones. These substances are formed as products of hydrolysis by enzymes When present in extracts from seeds, however, it is sometimes uncertain whether they formed original constituents of the seeds or resulted from hydrolysis.

As a result of the enzyme hydrolysis of proteins a mixture of various proteoses is usually produced (Chittenden and Mendel, 4) which can be separated by various methods, such as different solubilities in ammonium sulphate, alcohol, etc. The albumoses are soluble in water, salt solutions, dilute acids and alkalies They are all precipitated by complete saturation with ammonium sulphate, and some by half-saturation with the same salt On the whole, they give the general colour reactions of the proteins, and are precipitated by the protein precipitants, though some groups of proteoses show certain exceptions. Their solutions are not coagulated on boiling.

The peptones are the only proteins not precipitated by complete saturation with ammonium sulphate. They give the protein colour reactions and are precipitated by tannic acid and lead acetate

Expt. 126 *Separation and reactions of proteoses* Take 2 gms of the globulin edestin (prepared as in Expt 133) and put in a flask with 100 c c. of 0·2% hydrochloric acid and warm until as much as possible of the edestin goes into solution. Then cool and add 0 5 gm. of commercial pepsin add also a little toluol, shake and plug with cotton-wool Leave in an incubator at 38° C. for 4 days. (A control experiment should also be made with 100 c.c of 0 2 % hydrochloric acid and 0 5 gm of pepsin. Since pepsin itself gives a biuret reaction, a control is necessary for comparison in the next experiment) The preliminary changes in edestin hydrolysis are rapid, for it will be found that if the edestin solution is tested with sodium

chloride solution even after 24 hours in the incubator, no precipitate will be given with sodium chloride, in contrast with the copious precipitate given on the addition of salt solution to the unaltered acid solution of edestin (see also Expt 124) After four days, the incubated mixture is neutralized with dilute sodium carbonate solution, filtered and saturated while boiling with solid ammonium sulphate A precipitate of proteoses is formed, which can be gradually collected together as a sticky mass and removed with a glass rod Dissolve the precipitate in some hot water, filter and make the following tests

(*a*) *Xanthoprotein reaction* Add a few drops of nitric acid It is characteristic of most proteoses that a precipitate is formed which disappears on heating and reappears on cooling. In the case of the proteoses from edestin, only a slight precipitate may be given, but it is increased by adding a little sodium chloride solution The colour is intensified in the usual way by addition of ammonia

(*b*) *Millon's reaction* A positive result is given

(*c*) *Glyoxylic reaction* A positive result is given

(*d*) *Biuret reaction* A pink or pinkish-violet colour is given

(*e*) *Sulphur reaction* A positive result is given

(*f*) Add a little tannic acid solution A precipitate is formed

(*g*) Add a drop of copper sulphate solution A precipitate is formed

(*h*) Add a drop of strong acetic acid and then a couple of drops of potassium ferrocyanide A precipitate is formed which disappears on heating and reappears on cooling

(*i*) Boil some of the solution No coagulum is formed

Expt 127 *Detection of peptone* The saturated solution, from which the proteoses have been precipitated, is then filtered and to a measured quantity (about 5 c.c) twice the volume of 40 % sodium hydroxide is added and a drop of 1 % copper sulphate solution A pink colour appears, due to the presence of peptone. A test should be made with the control solution containing hydrochloric acid and pepsin only An adequate amount should be saturated with ammonium sulphate, filtered and 5 c c tested for peptone The reaction is less marked than in the actual hydrolytic product Concentrate the remainder of the peptone solution on a water-bath and pour off from the excess of ammonium sulphate crystals Filter and make the following tests (i) Xanthoproteic, (ii) Millon's, (iii) Glyoxylic, (iv) Tannic acid A positive result is obtained in each case

The Seed Proteins of certain Plants

The proteins present in the seeds of certain genera and species, upon which special investigations have been made, may now be considered

It should be borne in mind that there are always several proteins present in the seed. Some are reserve proteins of the cells of the endosperm or of the storage tissue of the cotyledons others are proteins of the protoplasm and nuclei of the tissues of the embryo and of the endosperm.

Proteins of Cereals (Graminaceae)

As far as investigations have gone it may be said that the starchy seeds of cereals are poor in albumins and globulins The chief reserve proteins belong to the peculiar group of prolamins, and a considerable portion also consists of glutelins

The grain of Wheat (*Triticum vulgare*) contains some proteose and a small percentage of an albumin, leucosin A globulin occurs only in very small amount The bulk of the protein consists of gliadin (a prolamin) and of glutenin (a glutelin). Nucleoproteins are present in the embryo, but there is no gliadin or glutenin (Osborne and Voorhees, 14)

Expt 128 *Extraction of the proteins of the Wheat grain* (*a*) *Extraction of albumin (leucosin) and proteose* Take 100 gms of white flour (the same quantity of wheat grains which have been ground in a coffee-mill may be used, but the extraction in this case is slower), put the ground mass in a large flask or beaker and add 250 c c of distilled water Let the mixture stand for 1–4 hrs , shaking occasionally Filter off some of the liquid, first through muslin and then on a filter-pump Reserve the residue on the filter and test the filtrate for proteins [Expt. 121, (*a*)-(*d*)]

Boil a second portion of the filtrate (after adding a drop or two of acetic acid) A precipitate of the albumin, leucosin, is formed Filter off this precipitate, cool the filtrate and make the protein tests again All the above tests are given by the proteose in solution in the case of the xanthoprotein, the precipitate disappears on heating and reappears on cooling (Expt 126) Also make the following special test for proteoses (Expt 126) Add a little potassium ferrocyanide solution and acetic acid A white precipitate is formed which disappears on heating and reappears on cooling

(*b*) *Extraction of the globulin* Take the residue of ground wheat and drain on a filter-pump Then extract with 250 c c of 10 % sodium chloride solution for 12–24 hrs Filter off, first through muslin, and then through paper on a filter-pump Put the extract to dialyze in a collodion dialyzer for 24 hrs (toluol should be added to the liquid in the dialyzer) Filter off the precipitate, which will be very slight, and dissolve it in a little 10 % sodium chloride (Though so little globulin is present, the experiment is instructive for comparison with the large amount of globulin obtained from many other seeds) Make the tests for protein [Expt. 121, (*a*)-(*d*)] with the solution (Millon's cannot be used on account of the presence of chlorides). Also try the effect of (i) boiling the sodium chloride solution coagulation is not complete, (ii) adding a little acid· a precipitate is formed as in the case of edestin

(*c*) *Extraction of gliadin* Take the wheat residue, which has been filtered from the sodium chloride solution, and add 250 c.c. of 95 % alcohol Warm on a water-bath and filter. Evaporate the filtrate, which contains gliadin, on a water bath (or better distil off the alcohol *in vacuo*) When reduced to about half its bulk, take a little of the filtrate and filter Divide this filtrate into two parts in test-tubes. To one add water to the other absolute alcohol A white precipitate of gliadin is formed in each case, since it is insoluble in both water and strong alcohol, though soluble in dilute alcohol The remainder of the gliadin extract is evaporated almost

to dryness, and then poured into a large volume of distilled water A milky precipitate of gliadin is formed which may be made to settle by adding a little solid sodium chloride and stirring Filter off the gliadin and dissolve in 10 % acetic acid With the solution make the tests for protein [Expt 121, (*a*)–(*d*)]

(*d*) *Extraction of glutenin* Take half the wheat residue from the alcoholic extraction, pound well in a mortar and extract again with warm alcohol and subsequently with water The residue must be free from water- and alcohol-soluble proteins as they are also soluble in alkalies Then extract the residue with 0 1 % caustic potash solution Filter off the extract which contains the glutenin To a portion of the filtrate add $\frac{N}{10}$ sulphuric acid drop by drop A precipitate of glutenin is formed Test the remainder of the filtrate for proteins [Expt 121, (*a*)–(*d*)]

The gliadin of wheat has the peculiar property of combining with water to form a sticky mass which binds together the particles of glutenin, the whole forming what is termed gluten It is this phenomenon which gives the sticky consistency and elastic properties to dough.

Expt 129 *To demonstrate the fact that gluten formation depends on the presence of gliadin* Take two small evaporating dishes Fill one with ordinary flour Fill the other with flour that has been extracted with 70 % alcohol for two or three days. (The alcohol is allowed to stand on the wheat in the cold It is then poured off, and more added, and the process repeated The flour is now dried again, first in air, then in the steam-oven and finally is ground in a mortar) A little water is added to each of the dishes and the flour worked up into a dough. This is then allowed to stand for half an hour The dough consists of gluten (gliadin and glutenin) to which the starch adheres Next take two beakers, fill with water, and over the top of each tie a muslin cover Place the two samples of dough on the muslin on the two beakers, and rub gently with a glass rod The starch will be washed away into the beakers In the case of the normal flour a sticky mass of gluten will remain In the other case there will be no gluten on account of the absence of gliadin To the suspension of starch in the beaker add some iodine solution, and it will turn a deep blue-black colour

In the Barley (*Hordeum vulgare*) grain, small percentages of an albumin, apparently identical with leucosin, and of a globulin, barley edestin, are present, together with some proteose The main protein is a prolamin, hordein, very similar to, but not identical with, gliadin There is no well-defined glutelin (Osborne, 9).

Expt. 130 *Extraction of the proteins of the Barley grain.* (*a*) *Extraction of the albumin and proteose* Grind up 100 gms of barley grains in a coffee-mill, or use preferably barley flour Add 250 c c of distilled water to the ground meal, and allow the mixture to stand for 1–4 hrs Filter off the extract, first through muslin and then through filter-paper The extract will contain a small quantity of the albumin, leucosin, and proteose. With the filtrate make the tests for proteins [Expt 121, (*a*)–(*d*)]

Boil a second portion of the filtrate. A white precipitate of the coagulated protein is formed Filter off the precipitate, cool the filtrate containing the proteose

and test for proteins All the tests will be positive in the case of the xanthoproteic, the precipitate disappears on heating and reappears on cooling, a characteristic of proteoses (Expt 126) Make also the special test for proteoses Add a little potassium ferrocyanide and acetic acid A white precipitate is formed which disappears on heating and reappears on cooling

(*b*) *Extraction of the globulin* To the barley residue, after extraction with water, add 250 c c of 10 % sodium chloride and allow the mixture to stand for 12–24 hrs Filter first through muslin and then filter-paper, and put the extract to dialyze for 24 hrs Filter off the precipitate of globulin which will have formed, and take it up into solution again in as small a quantity as possible of 10 % sodium chloride Make with the solution the following tests (i) The usual (except Millon's) tests for proteins [Expt 121, (*a*)–(*d*)] these will give positive results (ii) Boil a little of the solution imperfect coagulation will take place (iii) Add a little acid a precipitate is formed, as is usual with plant globulins

(*c*) *Extraction of the prolamin, hordein* The residue, after the sodium chloride extraction, is then extracted with 250 c c of warm 95 % alcohol Filter, and concentrate the filtrate on a water-bath (or better, distil *in vacuo*) After concentration, test a little filtered extract as follows pour a few drops into (1) absolute alcohol, (2) distilled water A white precipitate of hordein is produced in each case, since, like gliadin, it is insoluble in both strong alcohol and water, but soluble in dilute alcohol Then pour the whole extract into a large volume of water The protein is precipitated as a fine white suspension, but will settle out more readily if a little solid sodium chloride is added. Filter off the hordein, and dissolve in 1 % acetic acid Make the usual protein tests [Expt 121, (*a*)–(*d*)], there will be a positive result in each case

In the Rye (*Secale cereale*) grain there are small percentages of proteose, and of leucosin and edestin The greater part of the protein is gliadin, said to be identical with that in wheat

In the Maize (*Zea Mays*) grain there is apparently no true albumin, though there is some proteose There are small quantities of globulin, but the greater part of the protein is a prolamin, termed zein, and a glutenin (Osborne, 10).

Expt 131 *Extraction of proteins of the Maize grain* (*a*) *Extraction of proteins soluble in water* Grind up 100 gms of maize grains in a coffee-mill, or preferably use maize meal Add 250 c c of water and allow the mixture to stand 1–4 hrs Filter off, first through muslin, and then filter-paper The filtrate contains proteose and probably a little globulin which has gone into solution owing to the presence of salts in the seed Make the usual tests for protein [Expt. 121, (*a*)–(*d*)].

Boil another portion of the filtrate Some coagulation of protein will take place Filter, cool the filtrate and test for protein [Expt 121, (*a*)–(*d*)] Positive results will be given by the proteose present Make also the special test for proteoses. Add a little potassium ferrocyanide and acetic acid A white precipitate is formed, which disappears on heating and reappears on cooling

(*b*) *Extraction of globulin* The residue, after water extraction, is next treated with about 250 c c of 10 % sodium chloride solution for 12–24 hrs Filter, first

through muslin, and then through filter-paper. Dialyze the extract for 24 hrs Then filter off the precipitate of globulin which will have separated out, and dissolve in 10 % sodium chloride Make with the solution the following tests (i) The usual (except Millon's) tests for proteins [Expt 121, (*a*)-(*d*)] these will give positive results. (ii) Boil a little of the solution imperfect coagulation takes place (iii) Add a little acid the protein is precipitated.

(*c*) *Extraction of the prolamin, zein* The residue after salt extraction is then extracted with 250 c c of hot 95 % alcohol Filter, and concentrate the filtrate, which contains the zein, on a water-bath (or, better, distil *in vacuo*) Pour a few drops of the concentrated extract into (1) absolute alcohol, (2) distilled water As in the case of gliadin and hordein, a precipitate of zein will be formed Then pour the whole extract, after evaporating to a small bulk, into excess of distilled water, and add a little solid sodium chloride The precipitate of zein will slowly settle, and can be filtered off Zein is not readily soluble in acids and alkalies Hence Millon's and the xanthoproteic tests should be made on the solid material Zein does not contain the tryptophane nucleus To demonstrate this, the glyoxylic reaction should be made by shaking up some solid zein in reduced oxalic acid and adding sulphuric acid and mixing No purple colour is formed.

(*d*) *Extraction of glutenin* Take about half of the residue after the alcoholic extraction, pound in a mortar, and extract again with alcohol Then extract the residue with 0 1 % caustic potash solution. Filter off the extract which contains the glutenin To a portion of the filtrate add $\frac{N}{10}$ sulphuric acid drop by drop A precipitate of glutenin is formed. Test the remainder of the filtrate for proteins [Expt 121, (*a*)-(*d*)]

Proteins of Leguminous Seeds (*Leguminosae*).

In the Legumnosae, which are starchy seeds, the chief reserve proteins, as contrasted with those of cereals, are globulins The various proteins occurring may be enumerated as·

Legumin A globulin which forms the chief protein in the seeds of the Broad Bean (*Vicia Faba*), the Pea (*Pisum sativum*), the Lentil (*Ervum Lens*) and the Vetch (*Vicia sativa*) Legumin itself is soluble in water, but occurs as salts which are insoluble in water and soluble in saline solutions. Some portion can be extracted from the seed by water only.

Vicilin. A globulin occurring in smaller quantities than legumin and found only in the Pea, Bean and Lentil seeds

Phaseolin. A globulin forming the bulk of the protein of the Kidney Bean (*Phaseolus vulgaris*).

Conglutin. A globulin forming the bulk of the protein in Lupin (*Lupinus luteus*) seeds.

Legumelin. An albumin found in small quantities in the Pea, Broad Bean, Vetch and Lentil

Phaselin. An albumin found in small quantity in the seeds of the Kidney Bean (*Phaseolus vulgaris*)

Small quantities of proteoses are found in most of the above seeds

Expt 132. *Extraction of the proteins of the Pea* (Pisum sativum) (Osborne and Campbell, 11, 12, Osborne and Harris, 13) As we have seen (Expt 121), a certain amount of protein, including globulin, goes into solution when ground peas are extracted with water A more complete method of extraction is as follows Grind in a coffee-mill 20–30 gms of peas, add to the ground mass 50–60 c c of 10 % sodium chloride solution and allow the mixture to stand for 1–2 hrs Then filter off and saturate the filtrate with solid ammonium sulphate The globulins, legumin and vicilin, are precipitated out Filter off the precipitate, and then take up in dilute ammonium sulphate ($\frac{1}{10}$ saturated) and add saturated ammonium sulphate in the proportion of 150 c c to every 100 c c of the solution ($\frac{6}{10}$ saturation) The legumin is precipitated and can be filtered off Saturate the filtrate with ammonium sulphate the vicilin is precipitated and can be filtered off Dissolve up a little of each precipitate in 10 % sodium chloride, and boil The vicilin is coagulated, but the legumin is not Then dissolve up the remainder of the precipitates in dilute ammonium sulphate, and test both the solutions for protein by the usual reactions [Expt 121, (*a*)–(*d*)].

The albumin, legumelin, which occurs only in small quantities in the seeds, can be obtained by dialyzing a water extract of the ground peas until all the globulin is precipitated On filtering and heating the filtrate, a coagulum of legumelin is formed.

Proteins of fat-containing Seeds

Of the seeds which contain fat as a reserve material, those investigated have been found, in contrast to the cereals, to contain largely globulin as reserve protein. In many cases these globulins have been obtained in crystalline form after extraction from the plant

The Hemp-seed (*Cannabis sativa*) contains one of the best-known crystalline globulins, namely edestin Pure neutral edestin is insoluble in water but soluble in salt solutions In the presence of acid, however, edestin forms salts which are insoluble in salt solutions. Hence a solution of edestin in sodium chloride is precipitated by even small quantities of acids, and, conversely, a solution of edestin in acid is precipitated by small quantities of salt (Osborne, 8).

Expt 133 *Extraction and crystallization of edestin from Hemp-seed* Take 50 gms. of hemp-seed and grind in a coffee-mill Put the ground seed in a large evaporating dish and add 200 c c of 5 % sodium chloride solution Heat with a small flame and stir constantly A thermometer should be kept in the dish, and the liquid must not rise above 60° C Filter off, in small quantities at a time, keeping the solution in the dish warm On cooling, the edestin separates out from the filtrate more or less in

crystals To obtain better crystals, filter off the edestin that has been deposited, and pour the filtrate into a dialyzer, add a little toluol, and suspend the dialyzer in running water As soon as it is cloudy, examine the dialyzed solution for crystals under the microscope Add a little 5 % sodium chloride solution to the original precipitate of edestin in the filter Make with the filtrate the following tests (i) The tests for proteins [Expt 121, (*a*)-(*d*), except Millon's] (ii) Boil a little of the solution it is imperfectly coagulated (iii) Add a little acid edestin chloride is precipitated

In the Castor-oil seed (*Ricinus communis*) there is also present a globulin which can be obtained in a crystalline form by the method of Expt 134 In addition, there is present an albumin, ricin, which has peculiar toxic properties (Osborne, 8)

A well-crystallized globulin can be obtained from the Linseed (*Linum usitatissimum*), and a globulin, excelsin, from the Brazil nut (*Bertholletia excelsa*) also in crystalline or semi-crystalline form Similar globulins can be extracted from a number of other seeds, i e Coconut (*Cocos nucifera*), Sunflower (*Helianthus annuus*), Cotton-seed (*Gossypium herbaceum*), Mustard-seed (*Brassica alba*) and many others The fat is first removed from the ground seed by ether or benzene, the residue is then extracted with dilute sodium chloride and the extract dialyzed

Expt 134 *Extraction of the globulin from* Ricinus Weigh out about 50 gms of *Ricinus* seeds, take off the testas and pound in a mortar Extract the oil by the method given in Expt 82 After extracting the oil, grind up the residue again in a mortar, and then treat it with about twice its bulk of 10 % sodium chloride solution for 6–12 hrs Filter successively through muslin and filter-paper and dialyze the filtrate The globulin will be precipitated in semi-crystalline spheroids When the bulk of the globulin has separated out, filter off the precipitate, and dissolve it in as dilute a sodium chloride solution as possible Make the following tests with the solution (i) The tests for proteins [Expt 121, (*a*)-(*d*), except Millon's] (ii) Boil a little of the solution: the coagulation is not complete (iii) Add a little hydrochloric acid a precipitate is formed

Expt. 135 *Extraction of the globulin from Linseed* (Osborne, 7, 8) Weigh out about 50 gms of Linseed and grind it in a coffee-mill Extract the oil as in Expt. 82 Treat the residue with about twice its bulk of 10 % sodium chloride solution for 6–12 hrs Then filter through muslin and filter-paper, and dialyze the filtrate The globulin separates out in octahedra. Filter off the protein, and take up in dilute sodium chloride Test the solution as in the case of *Ricinus* globulin in the previous experiment

Expt 136 *Extraction of the globulin* (*excelsin*) *from the Brazil nut* (Osborne, 8) Weigh out about 100 gms of the nut, free from the testas, and, after pounding in a mortar, extract the oil by the usual method. Then proceed as in the two previous experiments. The protein separates out in semi-crystalline spheroids Filter off the precipitated excelsin, and dissolve in dilute sodium chloride solution Make with it the tests as for the globulins in the last two experiments.

THE AMINO-ACIDS

There is every reason to believe, since they always arise in hydrolysis of proteins, that amino-acids are universally distributed in the plant. It is, however, difficult to isolate and detect them, except in certain special cases, as, for instance, in germinating seeds when a large store of protein is being rapidly hydrolyzed and translocated The following is a short account of the occurrence of some of the amino-acids in the free state (see also p 120)

Valine has been isolated from seedlings of the Vetch (*Vicia*), Lupin (*Lupinus*) and Kidney Bean (*Phaseolus*) It is present in larger amounts in etiolated seedlings of Lupin than in the green plants.

Leucine is widely distributed. It has been isolated from seedlings of *Vicia*, Vegetable Marrow (*Cucurbita*), *Lupinus*, Pea (*Pisum*) and Goosefoot (*Chenopodium*) It has also been found in *Phaseolus*, Water Ranunculus (*Ranunculus aquatilis*), buds of Horse Chestnut (*Aesculus Hippocastanum*) and in small quantities in Potato tubers and other plants

Isoleucine has been extracted from seedlings of *Vicia sativa*

Aspartic acid The *amide* of this acid, i.e. **asparagin**,

$$CONH_2 \cdot CH_2\ CHNH_2\ COOH$$

is widely distributed in plants It is present in shoots of Asparagus from which it derives its name. It has also been extracted in very considerable quantities from etiolated seedlings of *Vicia*, Lupin, and from various plants such as Potato, Dahlia, Garden Nasturtium (*Tropaeolum*), *Cucurbita* and Sunflower (*Helianthus*)

Glutaminic acid. The *amide*, again, of this acid, i.e. **glutamine**,

$$CONH_2\ CH_2\ CH_2 \cdot CHNH_2\ COOH$$

is widely distributed It has been isolated from seedlings of *Cucurbita*, *Lupinus*, *Helianthus*, Castor-oil plant (*Ricinus*), Spruce Fir (*Picea excelsa*) and a number of Cruciferae

Arginine has been isolated from seedlings of *Lupinus*, *Cucurbita*, *Vicia*, and *Pisum* It is especially abundant in the seedlings of some Coniferae, i.e. *Picea excelsa*, Silver Fir (*Abies pectinata*) and Scotch Fir (*Pinus sylvestris*) It also occurs in roots and tubers, as for instance in those of the Turnip (*Brassica campestris*), Artichoke (*Helianthus tuberosus*), Chicory (*Cichorium Intybus*), Beet (*Beta vulgaris*), Potato and Dahlia, and in the inner leaves of the Cabbage (*Brassica oleracea*)

Lysine has been isolated from seedlings of *Lupinus*, *Vicia* and *Pisum*. Also from the inner leaves of the Cabbage and tubers of the Potato.

Phenylalanine has been isolated from seedlings of *Lupinus luteus*, *Vicia sativa* and *Phaseolus vulgaris*.

Tyrosine is very widely distributed. It is present in seedlings of *Vicia sativa*, *Cucurbita*, *Lupinus*, *Tropaeolum* and tubers of Potato, Turnip, Dahlia, Beet and Celery. Also in berries of Elder (*Sambucus*), in Clover (*Trifolium*), Bamboo (*Bambusa*) shoots and other plants.

Proline has been isolated in very small quantities from etiolated seedlings of *Lupinus albus*.

Histidine has been isolated from seedlings of *Lupinus* and tubers of Potato.

Tryptophane has been isolated from seedlings of *Lupinus albus* and *Vicia sativa*.

The Proteases.

We have seen in the previous pages that proteins can be hydrolyzed artificially with the intermediate production of proteoses and peptones, and the final production of a number of amino-acids. There is no doubt that this process of hydrolysis takes place in the living plant, and it is believed that the converse process, the synthesis of these proteins from amino-acids, also takes place in the cell.

There is evidence that this hydrolysis of proteins is catalyzed by certain enzymes which have been termed proteases. On analogy with other enzymes, we may suppose that these enzymes also catalyze the synthesis of the proteins.

It seems highly probable that the proteases are of two types:

1. Pepsin-like enzymes, which catalyze the hydrolysis of proteins to peptones, and, in all probability, the reverse process.

2. Erepsin-like enzymes, which catalyze the hydrolysis of albumoses and peptones to amino-acids, and, in all probability, the reverse process.

We now turn to the evidence for the existence of proteases. In autolysis (see p. 19) the hydrolytic activity of many enzymes is uncontrolled, and in the case of the proteins, the amino-acids are formed as end-products. Amino-acids are rarely present in plants in sufficient quantity to be detected readily, at any rate in small quantities of material, but if the tissues are put to autolyze at temperatures of 38–40° C.,

the amino-acids then accumulate and can be detected. Of all the amino-acids the one which is most readily identified is tryptophane If the autolyzed product is boiled, acidified and filtered to remove the remaining proteins, and, to the filtrate, bromine is added, drop by drop, the formation of a pink or purple colour will indicate the presence of free tryptophane, and hence it may be assumed that protein-hydrolysis has taken place Probably the formation of amino-acids in autolysis is a universal property of plant tissues, for tryptophane has been detected on autolysis of many different parts of plants Examples are the germinating seeds of the Bean (*Vicia Faba*), Scarlet Runner (*Phaseolus multiflorus*), Pea (*Pisum sativum*), Lupin (*Lupinus hirsutus*) and the Maize (*Zea Mays*) and in ungerminated seeds of the above, though less readily It is also said to be formed on autolysis of leaves of Spinach (*Spinacia*), Cabbage (*Brassica*), Nasturtium (*Tropaeolum majus*), Scarlet Geranium (*Pelargonium zonale*), Dahlia (*Dahlia variabilis*) and others also of fruits of Melon (*Cucumis Melo*), Cucumber (*Cucumis sativus*), Banana (*Musa sapientum*), Tomato (*Lycopersicum esculentum*) and others of bulbs of the Tulip (*Tulipa*), Hyacinth (*Hyacinthus orientalis*) and underground roots of Turnip (*Brassica*), Carrot (*Daucus Carota*) and Beet (*Beta vulgaris*) (Vines, 16–24, Blood, 3, Dean, 5, 6)

Expt 137 *The formation of tryptophane on autolysis of resting seeds* Grind up in a coffee-mill 15 gms. of Mustard (*Brassica alba*) seed. Transfer to a flask, and add 100 c c of distilled water and about 2 c c of toluol Plug the mouth of the flask with cotton-wool and put in an incubator for 3 days. Then filter off the liquid, boil the filtrate and add a few drops of acetic acid Filter off any precipitate formed, cool the filtrate and add bromine water *slowly and carefully drop by drop*, shaking well after each drop A pink or purple colour denotes the presence of tryptophane Excess of bromine will destroy the colour Then shake up with a little amyl alcohol The purple colour will be extracted by the amyl alcohol which will rise to the top of the water solution A control experiment should be made using 10 gms of seed which has been well boiled with water in an evaporating dish

It has been assumed that the formation of amino-acids from proteins on autolysis is the outcome of two processes, the hydrolysis of proteins to peptones by pepsins, and the hydrolysis of peptones to amino-acids by erepsins

The next point to be considered is the possibility of detecting these two classes of enzymes separately. If either the pulp, or water extract, of various plant tissues be added to peptone solution and allowed to incubate at 38° C., tryptophane can be readily detected after a day or two. This has been found to be true for the tissues of many seeds, seedlings, roots, stems, leaves and fruits (such as those already mentioned

above and others), the result indicates the wide distribution of an erepsin type of enzyme The detection of this enzyme is facilitated by the addition of the artificial supply of peptone

Expt 138 *The detection of erepsins in plants*

(*a*) *In resting seeds* Grind up 10 gms of seeds in a coffee-mill, and add 100 c.c of water, 0 2 gm of Witte's peptone and a little toluol Incubate for 2–3 days The following seeds may be used. Hemp (*Cannabis sativa*), Castor-oil (*Ricinus communis*), Pea (*Pisum sativum*), Scarlet Runner (*Phaseolus multiflorus*), Broad Bean (*Vicia Faba*) and fruit of Wheat (*Triticum vulgare*) Test for tryptophane Controls should be made in these and the following cases

(*b*) *In germinating seeds* Take 10 germinating peas, pound in a mortar, add 100 c c of distilled water, 0·2 gm of Witte's peptone, and a little toluol Incubate for 3 days Test for tryptophane

(*c*) *In leaves* Pound up a small cabbage leaf, add 100 c c of water, 0 2 gm of Witte's peptone and a little toluol Incubate for 3 days Test for tryptophane

(*d*) *In roots* Pound up about 20 gms of fresh carrot root. Add about 100 c c of water, 0 2 gm of Witte's peptone and a little toluol Incubate for 3 days Test for tryptophane

The pepsin type of enzyme is less readily detected It has long been known that the pitchers of the Pitcher-plant (*Nepenthes*) secrete an enzyme which digests fibrin. A few other cases of protein-digesting enzymes are well known, such as the so-called "bromelin" from the fruit of the Pine-apple (*Ananas sativus*), "cradein" from the latex and fruit of the Fig (*Ficus*) and "papain" from the fruit and leaves of the Papaw Tree (*Carica Papaya*) Such enzymes were formerly termed "vegetable trypsins" as they were thought to be of the type of animal trypsin which, alone, hydrolyzes proteins to amino-acids. On analogy with the results of research with other enzymes, it seems likely that "papain," "cradein" and "bromelin" are all mixtures of pepsin and erepsin. In addition to these better known cases, it has also been stated that fibrin is digested by extracts or pounded pulp of the fruits of the Cucumber and the Melon, the "germ" (embryo) of Wheat, the bulbs of Tulip and Hyacinth, the seedlings of the Bean, Pea, Scarlet Runner, Lupin and Maize, and the ungerminated seeds of the Pea, Lupin and Maize. These have also been shown to contain erepsin.

A separation of pepsin from erepsin has been achieved in the case of the seeds of the Hemp (*Cannabis sativa*) by means of the different solubilities of the two enzymes in water and salt solutions

Expt 139 *The extraction and the separation of the two enzymes, erepsin and pepsin, from Hemp-seed* (Cannabis sativa) (Vines, 22) Weigh out 50 gms of hemp-seed, grind it in a coffee-mill and extract with 250 c.c of 10% sodium chloride solution

Allow the mixture to stand all night and then filter Both operations should be carried out at as low a temperature as possible Measure the filtrate, and add acetic acid to the extent of 0 2 % A dense precipitate is formed Filter again, keeping as cool as possible

The acid filtrate contains the erepsin, but not the pepsin. Measure out 40 c c. into each of three small flasks, and add the following (i) 0 2 gm of Witte's peptone, (ii) the same, only boil the whole solution, (iii) 0 2 gm of carmine fibrin[1]. Add a little toluol to all three flasks, plug with cotton-wool, and incubate for three to four days. Test for tryptophane in flasks (i) and (ii), the first gives a marked reaction, the second little or no reaction The fibrin in (iii) will remain unaltered.

The precipitate produced by the acetic acid is then washed on the filter twice with 100 c c of 10 % sodium chloride solution, containing 0 2 % acetic acid, to remove traces of erepsin The precipitate is then treated with about 70 c.c of water, allowed to stand for a time, and then filtered The filtrate is divided into three equal portions Add the following respectively (i) 0 1 gm of carmine fibrin, (ii) the same, but the solution is boiled, (iii) 0 2 gm of Witte's peptone Add a little toluol to all three flasks, plug with cotton-wool and incubate for 3–4 days The fibrin will be seen to digest slowly in flask (i) (ii) will show no digestion, and (iii) will give no tryptophane reaction

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[1] Freshly washed and finely chopped fibrin is placed in carmine solution (1 gm carmine, 1 c c of ammonia, 400 c.c. of water) for 24 hrs Then strain off and wash in running water till washings are colourless

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CHAPTER IX

GLUCOSIDES AND GLUCOSIDE-SPLITTING ENZYMES

ATTENTION has been drawn to the fact (Chapters V and VII) that in the plant, compounds containing hydroxyl groups often have one or more of these groups replaced by the $C_6H_{11}O_5$— residue of glucose Such compounds are termed glucosides The substances in which this substitution most frequently occurs are of the aromatic class, and the glucosides may be regarded, on the whole, as ester-like compounds of carbohydrates with aromatic substances The non-sugar portion of the glucoside may vary widely in nature, and may be, for instance, an alcohol, aldehyde, acid, phenol, flavone, etc The sugar constituent is most frequently glucose, but pentosides, galactosides, mannosides and fructosides are also known Sometimes more than one monosaccharide takes part in the composition of the glucoside (These various relationships are shown in the accompanying table) The inclusion of all glucosides in a class is in a sense artificial· the character held in common (with very few exceptions) is that, on boiling with dilute acids, or, by the action of enzymes, hydrolysis takes place, and the glucoside is split up into glucose (or other sugar) and another organic constituent A number of compounds occurring as glucosides have already been dealt with, for example, the tannins and flavone, flavonol and anthocyan pigments, but, in these cases, the significance of the compounds lies rather in the nature of their non-sugar constituents than in the fact of their being glucosides

There are, however, a number of glucosides which have been grouped together and are more readily classified in this way than in any other. Some of them, doubtless, have come into prominence as glucosides on account of their association with well-known and specific enzymes, as, for instance, the glucoside amygdalin associated with the enzyme emulsin, and the glucoside sinigrin with the enzyme myrosin.

The hydrolyzing enzymes are by no means always specific, for *in vitro* one particular enzyme may be able to hydrolyze several glucosides. Many glucoside-splitting enzymes have been described, though there is no reason to suppose that each glucoside is only acted upon by an enzyme specific to that glucoside It is likely moreover that some of the different enzymes described will probably prove to be identical.

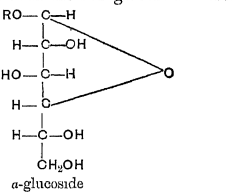
In some cases where more than one monosaccharide is attached to

the glucoside, the different sugar groups are removed separately by different enzymes (see later, emulsin, p 145)

The glucosides as a whole (except flavone, flavonol and anthocyan pigments) are colourless crystalline substances When extracting them from the plant, it is usually necessary to destroy the accompanying enzyme by dropping the material into boiling alcohol or some other reagent (see autolysis, p. 19)

In Chapter v it has already been mentioned that *d*-glucose exists in two stereoisomeric forms, the α and the β form

It was also pointed out that the glucosides can be classed either as α- or β-glucosides, according to whether the α or the β form of glucose combines with the non-glucose residue.



α-glucoside *β*-glucoside

Maltose, for instance, is regarded as an α-glucoside of *d*-glucose. It has been further shown that the enzyme maltase can only hydrolyze α-glucosides, whereas other enzymes, e.g the prunase component of emulsin, only act on β-glucosides.

The various glucosides considered in detail in this chapter together with some others are grouped under the following headings (Armstrong, 3) ·

Glucoside	Plant in which commonly found	Products of hydrolysis
		Alcohols
Coniferin	(Coniferae, *Beta*, *Asparagus*, *Scorzonera*)	Glucose + coniferyl alcohol
Populin	(*Populus*)	Glucose + saligenin + benzoic acid
Salicin	(*Salix*, *Populus*)	Glucose + saligenin
Syringin	(*Ligustrum*, *Syringa*, *Jasminum*)	Glucose + syringenin
		Aldehydes
Amygdalin	(*Prunus*, *Pyrus*)	Glucose + benzaldehyde + prussic acid
Dhurrin	(*Sorghum*)	Glucose + parahydroxybenzaldehyde + prussic acid
Linamarin	(*Linum*, *Phaseolus*)	Glucose + acetone + prussic acid

Glucoside	Plant in which commonly found	Products of hydrolysis
		Aldehydes (cont.)
Prulaurasin	(*Prunus*)	Glucose + benzaldehyde + prussic acid
Prunasin	(*Cerasus, Prunus*)	Glucose + benzaldehyde + prussic acid
Sambunigrin	(*Sambucus*)	Glucose + benzaldehyde + prussic acid
Vicianin	(*Vicia*)	Vicianose + benzaldehyde + prussic acid
		Acids
Gaultherin	(*Gaultheria, Spiraea*)	Glucose + methyl salicylate
Strophanthin	(*Strophanthus*)	Mannose + rhamnose + strophanthidin
		Phenols
Arbutin	(Ericaceae)	Glucose + hydroquinone
Hesperidin	(*Citrus*)	Glucose + rhamnose + hesperetin
Naringin	(*Citrus*)	G[illegible]
Phloridzin	(Rosaceae)	[illegible]
		Coumarin derivatives
Aesculin	(*Aesculus*)	Glucose + aesculetin
Fraxin	(*Fraxinus*)	Glucose + fraxetin
		Mustard-oils
Glucotropaeolin	(*Tropaeolum, Lepidium*)	Glucose + benzyl isothiocyanate + potassium hydrogen sulphate
Sinalbin	(*Brassica alba*)	G[illegible] l sulphate +
Sinigrin	(*Brassica nigra*)	Glucose + allyl isothiocyanate + potassium hydrogen sulphate
		Flavone and flavonol pigments
Apiin	(*Carum*)	Apiose + apigenin
Isoquercitrin	(*Gossypium*)	Glucose + quercetin
Lotusin	(*Lotus*)	Glucose + prussic acid + lotoflavin
Myricitrin	(*Myrica*)	Rhamnose + myricetin
Quercitrin	(*Quercus, Fraxinus, Thea*)	Rhamnose + quercetin
Robinin	(*Robinia*)	Rhamnose + galactose + kaempferol
Rutin	(*Ruta, Capparis, Polygonum*)	Glucose + rhamnose + quercetin
		Anthocyan pigments
Cyanin	(*Centaurea, Rosa*)	Glucose + cyanidin
Delphinin	(*Delphinium*)	Glucose + oxybenzoic acid + delphinidin
Malvin	(*Malva*)	Glucose + malvidin
Oenin	(*Vitis*)	Glucose + oenidin
Peonin	(*Paeonia*)	Sugar + peonidin
Pelargonin	(*Pelargonium, Centaurea*)	Glucose + pelargonidin
		Various constituents
Aucubin	(*Aucuba, Plantago*)	Glucose + aucubigenin
Digitalin	(*Digitalis*)	Glucose + digitalose + digitaligenin
Indican	(*Indigofera*)	Glucose + indoxyl

Cyanophoric Glucosides

The characteristic of these substances is that they yield prussic acid as one of the products of hydrolysis They are fairly widely distributed the following list (Greshoff, 15) includes most of the natural orders in which such glucosides occur Araceae, Asclepiadaceae, Berberidaceae, Bignoniaceae, Caprifoliaceae, Celastraceae, Compositae, Convolvulaceae, Cruciferae, Euphorbiaceae, Graminaceae, Leguminosae, Linaceae, Myrtaceae, Oleaceae, Passifloraceae, Ranunculaceae, Rhamnaceae, Rosaceae, Rubiaceae, Rutaceae, Saxifragaceae, Tiliaceae and Urticaceae

Amygdalin. This is one of the most important of the cyanophoric glucosides It occurs in the seeds of the bitter Almond (*Prunus Amygdalus*) but it appears to be almost entirely absent from the sweet or cultivated Almond It also occurs in the seeds of the other species of *Prunus*—the Plum (*P domestica*), the Peach (*P Persica*), etc.—of the Apple (*Pyrus Malus*) and the Mountain Ash (*P. Aucuparia*) It occurs sometimes in leaves, flowers and bark

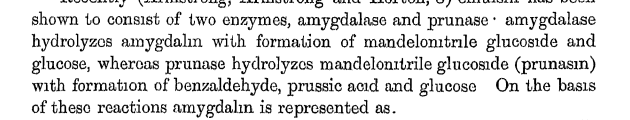
By the action of an enzyme, originally termed emulsin, which occurs in both the bitter and the sweet varieties of Almond, the glucoside is broken up as follows in two stages·

$$C_{20}H_{27}NO_{11} + H_2O = C_6H_{12}O_6 + C_{14}H_{17}NO_6$$
mandelonitrile glucoside (prunasin)

$$C_{14}H_{17}NO_6 + H_2O = C_6H_{12}O_6 + HCN + C_6H_5CHO$$
benzaldehyde

It should be noted that the sweet Almond contains emulsin although it is almost entirely free from amygdalin.

Recently (Armstrong, Armstrong and Horton, 8) emulsin has been shown to consist of two enzymes, amygdalase and prunase · amygdalase hydrolyzes amygdalin with formation of mandelonitrile glucoside and glucose, whereas prunase hydrolyzes mandelonitrile glucoside (prunasin) with formation of benzaldehyde, prussic acid and glucose On the basis of these reactions amygdalin is represented as.



Prunasin occurs naturally in the Bird Cherry (*Cerasus Padus*), and it is found that prunase may exist in a plant, e.g. Cherry Laurel (*P. Laurocerasus*), which does not contain amygdalase.

Prulaurasin (*laurocerasin*) is a glucoside occurring in the leaves of the Cherry Laurel (*Prunus Laurocerasus*). It has been represented as racemic mandelonitrile glucoside, prunasin being the dextro form

Sambunigrin is a glucoside occurring in the leaves of the Elder (*Sambucus nigra*). It has been represented as laevo mandelonitrile glucoside.

When tissues containing cyanophoric glucosides and their corresponding enzymes are submitted to autolysis, injury, or the action of chloroform, hydrolysis takes place (see autolysis, p 19). A rapid method (Mirande, 17, Armstrong, 5) for detecting the prussic acid is to insert paper dipped in a solution of sodium picrate into a tube containing the plant material together with a few drops of chloroform. In the presence of prussic acid the paper becomes first orange and finally brick-red owing to the formation of picramic acid

In addition to those previously mentioned there are other British plants, the leaves of which give off prussic acid on autolysis (presumably from cyanophoric glucosides), as for example the Columbine (*Aquilegia vulgaris*), Arum (*Arum maculatum*), Hawthorn (*Crataegus Oxyacantha*), Reed Poa (*Glyceria aquatica*), Bird's-foot Trefoil (*Lotus corniculatus*), Alder Buckthorn (*Rhamnus Frangula*), Black and Red Currant and Gooseberry (*Ribes nigrum, R rubrum, R Grossularia*), Meadow Rue (*Thalictrum aquilegifolium*) and the Common and Hairy Vetches (*Vicia sativa* and *V hirsuta*)

It has been shown (Armstrong, 7) that of the species *L. corniculatus* there is a variety (*L ulignosus*) (taller and growing in moister situations) which does not produce cyanophoric substances and hence does not give off prussic acid on autolysis.

Expt 140 *Method of detection of cyanophoric glucosides in the plant* Take three flasks in one put a whole leaf of the Cherry Laurel (*Prunus Lauvocerasus*). in the second a leaf which has been torn in pieces and then either pricked with a needle or pounded in a mortar in the third a leaf with a few drops of chloroform. Cork all three flasks, inserting with the corks a strip of sodium picrate paper (The paper is prepared in the following way. strips of filter-paper are dipped in a 1 % solution of picric acid, are then suspended on a glass rod and allowed to dry in air. Before using, the paper is moistened with 10 % sodium carbonate solution and is suspended in the moist condition just above the material to be examined In the presence of prussic acid, the paper first becomes orange-yellow, then orange and finally brick-red) In a short time the paper in the flask containing the leaf and chloroform will turn red. in the flask with the injured leaf, the reddening will take place rather more slowly, whereas in the case of the entire leaf, the paper will remain yellow

The above experiment may also be carried out, usually with success, on leaves of

the Columbine (*Aquilegia vulgaris*), the Arum (*Arum maculatum*) and plants of the Bird's-foot Trefoil (*Lotus corniculatus*) also with bitter almonds and apple pips, and young shoots of Flax (*Linum perenne*) In the case of the seeds, these may be used crushed, both with and without chloroform, the uninjured seed being used as a control.

Expt 141 *Preparation of amygdalin* Weigh out 100 gms of bitter almonds Remove the testas by immersing them for a short time in boiling water Then pound up the almonds well in a mortar and transfer to a flask Add about 200–300 c c of ether and allow the mixture to stand for 2–12 hours Filter off the ether and extract again with fresh ether The greater part of the fat will be removed in this way Then dry the residue from ether and, as rapidly as possible, extract twice or three times with boiling 90–98 % alcohol which removes the amygdalin The residue, after ether extraction, contains both amygdalin and emulsin, and, if allowed to stand, the emulsin will hydrolyze the amygdalin hence the necessity for rapid extraction with alcohol Evaporate the filtered alcoholic extract on a water-bath or, better, distil *in vacuo* to a small bulk Then add an equal volume of ether and allow the mixture to stand for a time The amygdalin separates out on standing Filter off the precipitate, dissolve in a little hot water and allow to crystallize in a desiccator

Expt 142 *Preparation of emulsin* (Bourquelot, 10) Weigh out 25 gms of sweet almonds (Bitter almonds can also be used The sweet variety is preferable, since from them the emulsin can be more readily prepared free from amygdalin) Plunge them for a moment into boiling water and remove the testas Pound thoroughly in a mortar, and extract the bulk of the oil with ether as in the last experiment Then grind up the residue with 50 c c of a mixture of equal parts of distilled water and water saturated with chloroform and allow the whole to stand for 24 hours. Filter by means of a filter-pump, and to the filtrate add glacial acetic acid (1 drop to 15 c c of the filtrate) whereby the protein is precipitated Again filter, and to the filtrate add 3–4 times its volume of 96–98 % alcohol. The emulsin is deposited as a white precipitate. Filter off the precipitate and dissolve it in about 100 c.c of cold distilled water

Expt. 143 (*a*) *To demonstrate the hydrolysis of amygdalin by emulsin* Into each of two flasks put 50 c c of a 1–3 % solution of amygdalin. To one flask add 25 c c of the emulsin solution prepared in the last experiment To the other flask add 25 c c of enzyme solution after it has been *well* boiled, and again boil the mixture after adding the enzyme Fit each flask with a cork and sodium picrate paper The paper in the flask containing the unboiled enzyme will rapidly turn red, the control remaining yellow. Unless both the enzyme and the amygdalin solution are well boiled in the case of the control, the paper may show reddening in time on account of traces of prussic acid present in both solutions

(*b*) *Simplified method for extraction of amygdalin and emulsin, and demonstration of hydrolysis of amygdalin by emulsin.* Take 12 bitter almonds. Remove the testas by immersing them for a short time in boiling water. Then pound up the almonds well in a mortar and transfer to a flask. Add about 50 c c of alcohol and heat to boiling on a water-bath. Filter off the extract, and evaporate it to dryness on a water-bath. The residue will contain amygdalin

Take six sweet almonds and remove the testas as before Pound in a mortar and transfer to a flask Add a little ether and allow to stand for a short time Pour off the ether, and add a little more which should again be poured off This removes some of the fat and makes extraction of the emulsin easier Then extract the residue with about 40 c c of distilled water and filter The filtrate contains the enzyme emulsin

Take 10 c c of the emulsin solution, and divide it into two portions in two test-tubes Boil one *well* (see Expt 143 *a*), and to both add equal quantities of a water extract of the amygdalin prepared above Cork the tubes and insert picric paper with the cork in each case

It has been found, as previously mentioned, that emulsin can hydrolyze other glucosides, as for instance, salicin (see pp. 48, 152) On hydrolysis, salicin splits up into salicylic alcohol (saligenin) and glucose. Salicin, itself, gives no colour with ferric chloride but saligenin gives a violet colour, and by means of this reaction the course of the hydrolysis can be followed

Expt 144 *To demonstrate the hydrolysis of salicin by emulsin* To 10 c c of a 1% solution of salicin in a test-tube add 10 c c of the emulsin solution prepared in Expt 142 or 143 As a control boil in a second test-tube another 10 c c of the emulsin solution and add 10 c c of salicin solution After about an hour, add to both test-tubes a few c c of strong ferric chloride solution A purple colour will be given in the first test-tube but no colour in the control The process of hydrolysis will be accelerated by placing the tubes in an incubator

A modification can be made as follows A second pair of test-tubes should be prepared as before and to both sufficient ferric chloride should be added to give a faint yellow tinge The unboiled mixture will gradually acquire a purple colour at ordinary temperature.

Other cyanophoric glucosides are dhurrin, phaseolunatin (linamarin), lotusin and vicianin

Dhurrin occurs in seedlings of the Great Millet (*Sorghum vulgare*) On hydrolysis it yields glucose, prussic acid and parahydroxybenzaldehyde ($C_6H_4 \cdot OH \cdot CHO$). It is hydrolyzed by emulsin

Phaseolunatin occurs in seeds of the wild plants of *Phaseolus lunatus* and in seedlings of Flax (*Linum*). It is associated with an enzyme which hydrolyzes it into acetone, glucose and prussic acid.

Lotusin occurs in *Lotus arabicus* On hydrolysis by an accompanying enzyme (lotase) it gives glucose, prussic acid and a yellow pigment, lotoflavin.

Vicianin occurs in the seeds of a Vetch (*Vicia angustifolia*). It is hydrolyzed by an accompanying enzyme into prussic acid, benzaldehyde and a disaccharide, vicianose.

Mustard-oil Glucosides

These are glucosides containing sulphur and they have been found chiefly among the Cruciferae. Sinigrin and sinalbin, the glucosides of mustard, have been most investigated

Sinigrin. This glucoside occurs in the seed of Black Mustard (*Brassica nigra*) and other species of *Brassica*. Also in the root of the Horse-radish (*Cochlearia Armoracea*) Sinigrin is hydrolyzed by the enzyme, myrosin (Guignard, 16, Spatzier, 18) (which occurs in the plant together with the glucoside), into allyl isothiocyanate, potassium hydrogen sulphate and glucose

$$C_{10}H_{16}O_9NS_2K + H_2O = C_3H_5NCS + C_6H_{12}O_6 + KHSO_4$$

Expt 145 *Extraction of sinigrin from Black Mustard* Weigh out 100 gms of Black Mustard seed. Grind the seed in a coffee-mill and afterwards pound in a mortar Heat 175 c c of 85 % alcohol to boiling in a flask on a water-bath and add the pounded mustard, and after boiling about ½ hour, filter and press out the alcohol Then put the dried cake of residue into 300 c c of water and allow the mixture to stand for 12 hours Press out the liquid and after filtering and neutralising with barium carbonate, concentrate *in vacuo* to a syrup Then extract with 90 % alcohol and filter On concentrating and exposing in a crystallizing dish, the sinigrin separates out in white needles

Sinalbin occurs in the seeds of White Mustard (*Sinapis alba*) By myrosin it is hydrolyzed to *p*-hydroxybenzylisothiocyanate, acid sinapin sulphate and glucose

$$C_{30}H_{42}O_{15}N_2S_2 + H_2O = C_6H_{12}O_6 + C_7H_7ONCS + C_{16}H_{24}O_5NHSO_4$$

Expt 146 *Extraction of sinalbin from White Mustard* Weigh out 100 gms of White Mustard seed Grind and pound well and extract the fat with ether Then extract with twice its weight of 85–90 % alcohol several times and well press out the alcohol The extract is evaporated to half its bulk and filtered. On cooling the sinalbin separates out in crystals

Expt 147 *Preparation of myrosin.* Weigh out 50 gms. of White Mustard seed and grind in a coffee-mill Add 100 c c of water and allow the mixture to stand for 12 hours Then filter and allow the filtrate to run into 200 c.c. of 95–98 % alcohol A white precipitate is formed which contains the myrosin Filter off the precipitate and wash on the filter with a little ether

Expt. 148 *Action of myrosin on sinigrin* Put into two test-tubes equal quantities of a solution of the sinigrin prepared in Expt. 145. Dissolve some of the myrosin prepared in the last experiment in water and divide the solution into two parts Heat one part to boiling and then add the two portions respectively to the two test-tubes of sinigrin Plug both test-tubes with cotton-wool. After about ½ hour a strong pungent smell of mustard oil, allyl isothiocyanate, will be detected in the unboiled tube.

A more simple method of demonstrating the action of myrosin is as follows

Pound about 5 gms of Black Mustard seed in a mortar and then boil with water Some mustard oil will be formed before the myrosin is destroyed, so that boiling should be continued until no pungent odour can be detected Then filter and cool the solution and divide into two parts To one add some myrosin solution To the other an equal quantity of boiled enzyme solution After ½ hour the smell of allyl isothiocyanate should be detected in the unboiled tube.

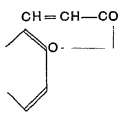
Saponins

These substances are very widely distributed, being found in the orders Araliaceae, Caprifoliaceae, Combretaceae, Compositae, Cucurbitaceae, Gramineae, Guttiferae, Lecythidaceae, Leguminosae, Liliaceae, Loganiaceae, Magnoliaceae, Myrtaceae, Oleaceae, Piperaceae, Pittosporaceae, Polemoniaceae, Polygalaceae, Primulaceae, Proteaceae, Ranunculaceae, Rhamnaceae, Rosaceae, Rutaceae, Saxifragaceae, Thymelaeaceae and the majority of the orders of the cohort Centrospermae On hydrolysis with dilute mineral acids the saponins yield various sugars—glucose, galactose, arabinose, rhamnose—together with other substances termed sapogenins

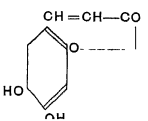
The saponins are mostly amorphous substances readily soluble in water (except in a few cases) giving colloidal solutions These solutions froth on shaking, and with oils and fats they produce very stable emulsions By virtue of this property they have been used as substitutes for soap The Soapwort (*Saponaria*) owes its name to the fact that the root contains a saponin

Coumarin Glucosides

These substances are hydroxy derivatives of coumarin, which itself may be represented as



Aesculin is one of the best known of these glucosides. It occurs in the bark of the Horse Chestnut (*Aesculus Hippocastanum*). On hydrolysis with dilute acids it yields glucose and aesculetin, the latter being represented as



Aesculin is characterized by giving in water solution a blue fluorescence which can be detected even in great dilution. The fluorescence is increased in alkaline, and decreased in acid, solution.

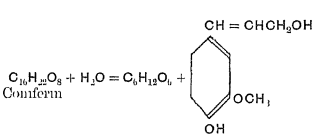
Expt 149 *Demonstration of the presence of aesculin in* Aesculus *bark* Strip off the bark from some young twigs of *Aesculus* and boil in a little water in an evaporating dish Filter and pour the filtrate into excess of water in a large vessel A blue fluorescent solution will be formed.

Glucosides of Flavone, Flavonol and Anthocyan Pigments

These substances have already been considered in Chapter VII

Glucosides of various Composition

Coniferin This glucoside occurs in various members of the Coniferae and also in *Asparagus* On hydrolysis with mineral acids or emulsin, it breaks up as



Coniferyl alcohol

Arbutin This glucoside is found in the leaves of the Bearberry (*Arctostaphylos Uva-ursi*), *Pyrola*, *Vaccinium*, and other Ericaceae and also of the Pear (*Pyrus communis*).

On hydrolysis with acids arbutin yields hydroquinone and glucose

$$C_{12}H_{16}O_7 + H_2O = C_6H_6O_2 + C_6H_{12}O_6$$

the same hydrolysis is brought about by the enzyme emulsin.

It has been suggested that the darkening of leaves of the Pear (Bourquelot and Fichtenholz, 11, 12, 13) either on autolysis or injury, or at the fall of the leaf, is due to the hydrolysis of the arbutin by a glucoside-splitting enzyme in the leaf, and subsequent oxidation of the hydroquinone so formed by an oxidase.

Expt 150. *Extraction of arbutin from leaves of the Pear* (Pyrus communis) Weigh out 100 gms. of fresh leaves (without petioles) Tear the leaves into small pieces and drop them as quickly as possible into about 500 c c of boiling 96–98 % alcohol in a flask Boil for about 20 mins., adding more alcohol if necessary Then filter off the alcohol and pound up the leaf residue in a mortar and extract again with

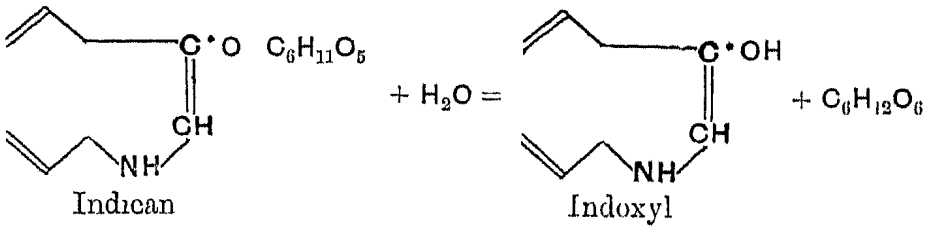
boiling alcohol Filter and distil off the alcohol from the extract *in vacuo* Extract the residue with 100–200 c c of hot water and filter Warm the filtrate and precipitate with lead acetate solution until no more precipitate is formed This removes flavones, tannins, etc but the arbutin is not precipitated Filter and pass sulphuretted hydrogen into the filtrate to remove any excess of lead acetate Filter and concentrate the filtrate *in vacuo* to a syrup Then extract twice with small quantities of ethyl acetate. Concentrate the ethyl acetate on a water-bath and cool A mass of crystals of arbutin will separate out This should be filtered off on a small filter, and recrystallized from ethyl acetate Take up a little of the purified glucoside in water and add a drop or two of ferric chloride solution A blue coloration will be given

Salicin This substance occurs in the bark of various species of Willow (*Salix*) and Poplar (*Populus*) also in the flower-buds of the Meadow-Sweet (*Spiraea Ulmaria*) On hydrolysis with acids, or on treatment with emulsin, salicin is decomposed into saligenin or salicylic alcohol and glucose

$$C_{13}H_{18}O_7 + H_2O = C_6H_4OH \cdot CH_2OH + C_6H_{12}O_6$$

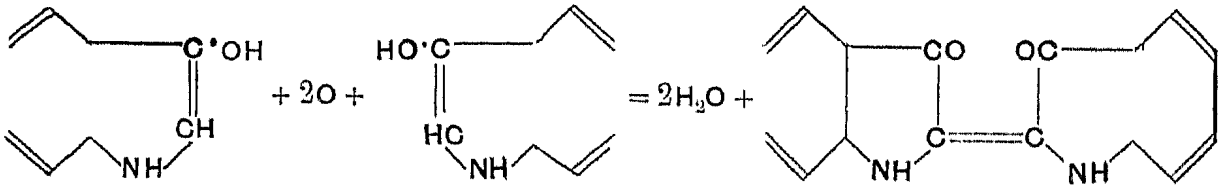
Saligenin gives a violet colour with ferric chloride solution and in this way the progress of the reaction can be demonstrated (see also p 148)

Indican (see also p 115) This glucoside occurs in shoots of the so-called "Indigo Plants," *Indigofera Anil, I erecta, I tinctoria, I sumatrana* also in the Woad (*Isatis tinctoria*), in *Polygonum tinctorium* and species of the Orchids, *Phajus* and *Calanthe* When boiled with acid or hydrolyzed by an enzyme contained in the plant, it gives glucose and indoxyl



Indican Indoxyl

The colourless indoxyl can be oxidized either artificially or by an oxidase contained in the plant to a blue product, indigotin or indigo



Indoxyl Indoxyl Indigo

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CHAPTER X

THE PLANT BASES

THERE are present in plants a number of substances which form group, and which may be termed nitrogen bases, or natural base These substances are of various constitution but they have the proper in common of forming salts with acids by virtue of the presence primary, secondary, or tertiary amine groupings Such groupings conf a basic property upon a compound and, as a result, salts are form with acids on analogy with the formation of ammonium salts

$$NH_3 + HCl = NH_4Cl \ (NH_3 \quad HCl)$$

$$CH_3NH_2 + HCl = CH_3NH_2 \cdot HCl$$

methylamine

$$(CH_3)_2\,NH + HCl = (CH_3)_2\,NH \cdot HCl$$

dimethylamine

$$(CH_3)_3\,N + HCl = (CH_3)_3\,N \quad HCl$$

trimethylamine

The hydrogen atoms of ammonia can also be replaced by groups greater complexity, as will be seen below.

Complex ring compounds in which nitrogen forms part of the ri are termed heterocyclic, such as the alkaloids, purines and some amin for instance pyrrolidine (see below)

The plant bases can be conveniently classified into four groups a this is also to a large extent a natural grouping. They are·

1 Amines } Simpler natural bases.
2 Betaines }
3 Alkaloids
4. Purine bases.

The first two groups have been termed the simpler natural bas They are much more widely distributed in the vegetable kingdom th the alkaloids and purins, since they have probably much more significar in general metabolism The isolation of the simpler bases is a matter much greater difficulty than that of the alkaloids. the former are solul in water but insoluble in ether and chloroform, and so are not read separated from other substances The alkaloids, however, occur in t plant as salts of acids and if the plant material is made alkaline the fi bases can be extracted with ether or chloroform

The betaines are amino-acids in which the nitrogen atom is completely methylated, and, with one or two exceptions, this grouping does not occur in the true alkaloids The betaines have only feebly basic properties.

The alkaloids, in contrast to the simpler natural bases, are rather restricted in their distribution, many being limited to a few closely related species or even to one species

The purine bases are a small group of substances intimately related to each other and to uric acid

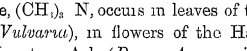
Amines

Methylamine, $CH_3.NH_2$, occurs in the Annual and Perennial Dog's Mercury (*Mercurialis annua* and *M perennis*) and in the root of the Sweet Flag (*Acorus Calamus*)

Trimethylamine, $(CH_3)_3N$, occurs in leaves of the Stinking Goosefoot (*Chenopodium Vulvaria*), in flowers of the Hawthorn (*Crataegus Oxyacantha*) and Mountain Ash (*Pyrus Aucuparia*), and in seeds of *Mercurialis annua*

Putrescine, $NH_2(CH_2)_4NH_2$, occurs in the Thorn Apple (*Datura*) and tetramethylputrescine in a species of Henbane (*Hyoscyamus muticus*)

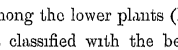
Hordenine occurs in germinating Barley grains It is represented as



Pyrrolidine is said to occur in small quantities in leaves of the Carrot (*Daucus Carota*) and Tobacco (*Nicotiana*) leaves It is represented as

Other amines occur among the lower plants (Fungi).

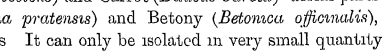
Choline is sometimes classified with the betaines. It is however intimately connected with the phosphatides (compounds of the fatty acids with phosphoric acid and nitrogen) which is not the case with the betaines. It may be represented as:



Choline is very widely distributed in plants It is a constituent of the phosphatide, lecithin, and is probably thereby a constituent of all living cells. It has been found in seeds of the Bean (*Vicia Faba*), Pea (*Pisum sativum*), *Strophanthus*, Oat (*Avena sativa*), Cotton (*Gossypium herbaceum*), Beech (*Fagus sylvatica*), Fenugreek (*Trigonella Foenum-graecum*) and Hemp (*Cannabis sativa*) in seedlings of Lupins, Soy beans, Barley and Wheat in Potatoes and Dahlia tubers and in the subterranean parts of Cabbage (*Brassica napus*), Artichoke (*Helianthus tuberosus*), *Scorzonera hispanica*, Chicory (*Cichorium Intybus*), Celery (*Apium graveolens*) and Carrot (*Daucus Carota*) aerial parts of Meadow Sage (*Salvia pratensis*) and Betony (*Betonica officinalis*), and many other tissues It can only be isolated in very small quantity

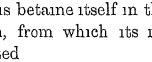
Betaines

The betaines, as previously stated, are amino-acids in which the nitrogen atom is completely methylated Most betaines crystallize with one molecule of water, thus betaine itself in this condition probably has the following constitution, from which its relationship to glycine or aminoacetic acid is indicated



Betaine or hydroxytrimethyl-aminoacetic acid — Aminoacetic acid

When dried above 100° C, the betaines lose water and are represented as cyclic anhydrides, thus betaine becomes



The individual betaines, probably on account of their close connexion with proteins, are more widely distributed than the individual alkaloids. Further investigation may show an even more general distribution of betaines.

Betaine or trimethylglycine occurs in all species of Chenopodiaceae so far examined including the sugar Beet (*Beta vulgaris*) from which it derives its name, in some genera only of the Amarantaceae; in the "Tea Plant" (*Lycium barbarum*) in seeds of Cotton (*Gossypium herbaceum*), Sunflower (*Helianthus annuus*) and Oat (*Avena sativa*) in tubers of Artichoke (*Helianthus tuberosus*), shoots of Bamboo (*Bambusa*), leaves of Tobacco (*Nicotiana Tabacum*) and in malt and wheat germs.

Stachydrine, though a betaine, is included by most writers among the alkaloids, and this classification has been followed here (see p. 161), it is probably a derivative of proline (see p. 121).

Betonicine, $C_7H_{13}O_3N$, is also, like stachydrine, found in the Betony (*Betonica officinalis*) It is a derivative of oxyproline

Hypaphorine or trimethyltryptophane, $C_{14}H_{18}O_2N_2$, occurs in the seeds of a tree, *Erythrina Hypaphorus*, which is grown for shade in Coffee plantations

Trigonelline, like stachydrine, is usually classed with the alkaloids (see p 160) but it should probably be included among the betaines on account both of its structure and of its wide distribution

Other betaines, **trimethylhistidine, ergothioneine**, occur in the Fungi

Alkaloids

The plant alkaloids, so-called because of their basic properties, have attracted considerable attention on account both of their medicinal properties and, in many cases, their intensely poisonous character They were also the plant bases to be first investigated. As previously mentioned they are not widely distributed, some being, as far as is known, restricted to one genus, or even species. Moreover, several closely related alkaloids are frequently found in the same plant. The orders in which they largely occur are the Apocynaceae, Leguminosae, Papaveraceae, Ranunculaceae, Rubiaceae and Solanaceae.

The alkaloids may be present in solution in the cell-sap in the young tissues, but in older and dead tissues they may occur in the solid state, they may be found throughout the plant or more abundantly in the seed, fruit, root or bark (quinine)

The alkaloids are, as a rule, insoluble in water, but soluble in such reagents as alcohol, ether, chloroform, etc. The majority are crystalline solids which are not volatile without decomposition, but a few, for example coniine, nicotine, which contain no oxygen, are volatile liquids

The alkaloids occur in the plant as a rule as salts of various organic acids, such as malic, citric, succinic and oxalic, and sometimes with an acid peculiar to the alkaloid with which it is united (e.g. quinic acid in quinine and meconic acid in opium). Artificial salts, i.e. sulphates, chlorides and nitrates, are easily prepared and are readily soluble in water, and from these solutions the free base is precipitated again on addition of alkali

The alkaloids themselves belong to various classes of compounds, though the basic character always preponderates. Thus, for example, piperine is an amide and can be hydrolyzed into the base piperidine and piperic acid atropine is an ester made up of the base tropine and tropic acid

Various methods are employed for the extraction of alkaloids but the exact course of events depends on the alkaloid in question. On the whole the method is either to treat the plant material with alkali and then extract the free alkaloid with ether or chloroform and finally purify by making a salt again, or to extract the alkaloid from the plant with dilute acid, set free the insoluble, or difficultly soluble, base with alkali, and then prepare a salt of the base

Though individual alkaloids have distinctive reactions, the group as a whole has certain reactions in common, namely the precipitation by the so-called "alkaloidal reagents" These reagents are tannic, phosphotungstic, phosphomolybdic and picric acids, also potassium-mercuric-iodide solution and iodine in potassium iodide solution

Expt 151 *General reactions of alkaloids* Make a 0 5 % solution of quinine sulphate in warm water and add a few drops of each of the following reagents

(*a*) Tannic acid solution A white precipitate is formed

(*b*) Mercuric iodide in potassium iodide solution [Brucke's reagent 50 gms of potassium iodide in 500 c c water are saturated with mercuric iodide (120 gms) and made up to 1 litre] A white precipitate is formed

(*c*) Phosphotungstic acid (50 gms of phosphotungstic acid and 30 c.c. of conc sulphuric acid are dissolved in water and made up to a litre) A white precipitate is formed

(*d*) Iodine in potassium iodide solution A brown precipitate is formed.

(*e*) Picric acid solution A yellow precipitate is formed

Expt 152. *Extraction of the free base from quinine sulphate* Add strong sodium carbonate solution drop by drop to some of the quinine sulphate solution until a white precipitate of quinine is formed Then add ether and shake up in a separating funnel The precipitate will disappear as the quinine passes into solution in the ether. Separate off the ethereal solution and let it evaporate in a shallow dish The quinine is deposited Take up the quinine again in dilute sulphuric acid and test the solution with the alkaloidal reagents

The alkaloids are classified into five groups according to the nucleus which constitutes the main structure of the molecule. These five groups are

1 The pyridine group
2 The pyrrolidine group.
3. The tropane group.
4 The quinoline group.
5 The isoquinoline group.

Pyridine

Pyrrole

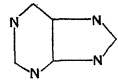
Tropane

Quinoline

Isoquinoline

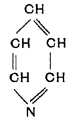
Pyrimidine

Imnazole


Purine

1. *The pyridine alkaloids*

These are, as the name implies, derivatives of pyridine. (Pyridine is a colourless liquid which boils at 115° C It is a strong base and forms salts with acids)

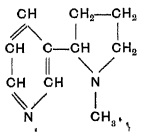

Pyridine

The more important members of this group are. arecoline, coniine, nicotine, piperine and trigonelline.

Arecoline occurs in the "Betel Nut" which is the fruit of the Areca Palm (*Areca Catechu*).

Coniine occurs in all parts of the Hemlock (*Conium maculatum*), but more especially in the seed.

Nicotine occurs in the leaves of the Tobacco plant (*Nicotiana Tabacum*). It is a colourless oily liquid which is intensely poisonous. Its constitution may be represented as.



It is readily soluble in water and organic solvents.

Expt 153 *Extraction and reactions of nicotine* Weigh out 100 gms of plug tobacco and boil up the compressed leaves with water in an evaporating dish or in a saucepan Filter off the extract and concentrate on a water-bath The concentrated solution is made alkaline with lime and distilled from a round-bottomed flask fitted with a condenser, the flask being heated on a sand-bath The distillate has an unpleasant smell and contains nicotine in solution Test the solution with the alkaloidal reagents employed in Expt 151 A precipitate will be obtained in each case

The nicotine can be obtained from solution in the following way. Acidify the aqueous distillate with oxalic acid and concentrate on a water-bath Make the concentrated solution alkaline with caustic soda, pour into a separating funnel and shake up with ether. Separate the ethereal extract and distil off the ether The nicotine is left behind as an oily liquid which oxidizes in air and turns brown The alkaloidal tests should be made again with the extracted nicotine

Piperine occurs in various species of Pepper (*Piper nigrum*) The fruit, which is gathered before it is ripe and dried, yields a black pepper, but if the cuticle is first removed by maceration, a white pepper. Piperine is a white solid which is almost insoluble in water but soluble in ether and alcohol

Expt 154. *Extraction and reactions of piperine* Weigh out 100 gms of black pepper. Put it into an evaporating dish, cover well with lime-water and heat with constant stirring for 15–20 minutes Then evaporate the mixture completely to dryness on a water-bath Grind up the residue in a mortar, put it into a thimble and extract with ether in a Soxhlet Distil off the ether and take up the residue in hot alcohol from which the piperine will crystallize out With an alcoholic solution make the following tests

(*a*) Add the alkaloidal reagents mentioned in Expt 151 and note that a precipitate is formed in each case

(*b*) Pour a little of the solution into water and note that the piperine is precipitated as a white precipitate

(*c*) To a little solid piperine in a white dish add some concentrated sulphuric acid It dissolves to form a deep red solution

Trigonelline occurs in the seeds of the Fenugreek (*Trigonella Foenum-graecum*), Pea (*Pisum sativum*), Bean (*Phaseolus vulgaris*), *Strophanthus hispidus*, Hemp (*Cannabis sativa*) and Oat (*Avena sativa*). It is also found in the Coffee Bean (*Coffea arabica*), in tubers of *Stachys tuberifera*, Potato and Dahlia and in roots of *Scorzonera hispanica*. It is really a betaine (see p 157).

2. *The pyrrolidine alkaloids.*

These are derivatives of pyrrolidine, of which the mother substance is pyrrole. (Pyrrolidine is a liquid boiling at 91° C. It is a strong base and forms stable salts with acids.)

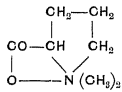
Pyrrole

Pyrrolidine

These alkaloids form a small group containing:

Hygrine and **cuskhygrine** which occur in Coca leaves (*Erythroxylon Coca*)

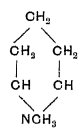
Stachydrine which occurs in tubers of *Stachys tuberifera* and leaves of the Orange Tree (*Citrus Aurantium*) and in various other plants (*Betonica*) The formula is



from which it is seen that it is really a betaine (see p 157)

3. *The tropane alkaloids.*

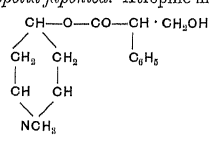
These are derivatives of tropane, which may be regarded as formed from condensed piperidine and pyrrolidine groupings. (Tropane is a liquid boiling at 167° C)



Tropane

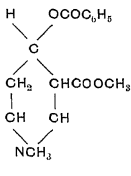
The alkaloids in this group are limited to four natural orders and are as follows.

Solanaceae. **Atropine** occurs in the root and other parts of the Deadly Nightshade (*Atropa Belladonna*), the Thorn Apple (*Datura Stramonium*) and *Scopolia japonica*. Atropine may be represented as



Hyoscyamine occurs in the Henbane (*Hyoscyamus niger*), *H. muticus* and also in the Mandrake (*Mandragora*)

Erythroxylaceae **Cocaine** and **tropacocaine** occur in Coca leaves (*Erythroxylon Coca*) together with smaller quantities of allied alkaloids. Cocaine has the formula

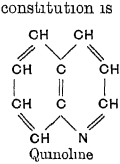


Punicaceae **Pelletierine** and other allied alkaloids occur in the root and stem of the Pomegranate Tree (*Punica Granatum*)

Leguminosae **Sparteine** occurs in the Broom (*Spartium scoparium*): **lupinine** in the yellow and black Lupins (*Lupinus luteus* and *L. niger*) and **cytisine** in the Laburnum (*Cytisus Laburnum*)

4 *The quinoline alkaloids*

These are derivatives of quinoline. (Quinoline is a colourless liquid which boils at 239° C.) Its constitution is



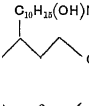
Quinoline

These alkaloids form two natural groups, (*a*) the cinchona alkaloids, i.e. quinine, cinchonine and allied forms, and (*b*) the strychnine alkaloids, i.e. strychnine and brucine.

Quinine occurs in the bark of various species of the genus *Cinchona* (Rubiaceae) which are trees, originally natives of S. America, but now cultivated on a large scale in Ceylon, Java and India. The species employed are *C. Calisaya, Ledgeriana, officinalis, succirubra*. The yellow bark of *Calisaya* has the highest percentage, i.e. 12 %, of alkaloid.

Quinine is a white solid which crystallizes in long needles containing water of crystallization. It is very slightly soluble in cold water, more so in hot but readily soluble in alcohol, ether and chloroform. With

acids it forms salts, which are soluble in water, the sulphate being commonly employed in medicine Quinine is said to have the following constitution



Expt 155 *Extraction and reactions of quinine* Mix 20 gms of quicklime with 200 c c of water in a basin and then add 100 gms of powdered Cinchona bark. Stir together well and then dry the mixture thoroughly on a water-bath, taking care to powder the lumps The dried mixture is then extracted in a Soxhlet apparatus with chloroform The chloroform extract is then shaken up in a separating funnel with 25 c c of dilute sulphuric acid The chloroform layer is run off and again extracted with water. The sulphuric acid and water extracts are mixed together and neutralized with ammonia The liquid is evaporated on a water bath until crystals of quinine sulphate begin to separate out With the quinine sulphate the following tests should be made (It is better to use a solution of the hydrochloride prepared by adding a few drops of hydrochloric acid to the sulphate solution)

(*a*) Test with the alkaloidal reagents of Expt 151

(*b*) Add to a little of the solution some bromine water and then some ammonia A green precipitate is formed which gives a green solution with excess of ammonia

(*c*) Dissolve a little of the solid quinine sulphate in acetic acid and pour into a large volume of water A blue opalescence is produced which is characteristic of quinine

Cinchonine occurs together with quinine in Cinchona bark It is very similar in constitution to quinine, the latter being methoxy-cinchonine

Strychnine and **brucine** occur in the seeds of Nux Vomica (*Strychnos Nux-vomica*) and St Ignatius' Bean (*S. Ignatii*).

Expt 156 *Tests for strychnine.* Add a little concentrated sulphuric acid to a small quantity of strychnine in an evaporating dish and then add a small amount of powdered potassium bichromate A violet coloration is produced which changes to red and finally yellow.

Curarine, the South American Indian Arrow poison, occurs in several species of *Strychnos* (*S toxifera* and others).

5. *The isoquinoline alkaloids.*

These can be divided into two groups· (*a*) the opium alkaloids and (*b*) the berberine alkaloids

The opium alkaloids again fall into two classes: (1) the papaverine

group which includes **papaverine, laudanosine, narceine, narcotine** and others, and (2) the morphine group including **morphine, apomorphine, codeine, thebaine** and others

Opium is the dried latex obtained by making incisions in the capsules of the Opium Poppy (*Papaver somniferum*)

Allied to the papaverine group is **hydrastine** which occurs in the root of *Hydrastis canadensis* (Ranunculaceae)

The constitution of all these alkaloids is very complex

Expt 157 *Tests for morphine*

(*a*) Add a little ferric chloride solution to a solution of a morphine salt. A deep blue coloration is formed

(*b*) Dissolve some morphine in concentrated sulphuric acid and then after standing about 15 hrs add concentrated nitric acid A deep blue-violet colour is produced which afterwards changes to red

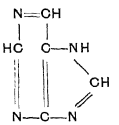
Berberine occurs in the root of the Barberry (*Berberis vulgaris*) and is also found in isolated genera in Anonaceae, Menispermaceae, Papaveraceae, Ranunculaceae and Rutaceae

Corydaline occurs in *Corydalis cava* (Fumariaceae)

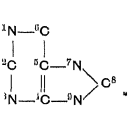
Many other alkaloid substances have been isolated from a large number of different plants, but since the constitution of most of them is unknown, they have not been classified

Purine Bases

These substances, as indicated, have a heterocyclic ring structure and are derivatives of purine. the atoms of the ring are numbered in the order indicated below



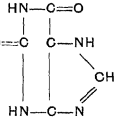
Purine



Purine itself is a crystalline basic compound (m.p. 211–212° C.) which forms salts with acids It is composed of two rings, the pyrimidine and the imiazole the latter grouping also occurs in histidine (see p. 121)

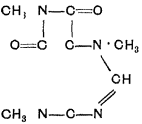
The chief purine bases which occur in plants are xanthine, caffeine, theobromine, guanine, hypoxanthine and adenine

Xanthine may be regarded as 2, 6-dioxypurine



It is widely distributed in plants and has been found in leaves of the Tea plant (*Thea sinensis*), in the sap of the Beetroot (*Beta*) and in various seedlings

Caffeine or **theine** is 1, 3, 7-trimethylxanthine



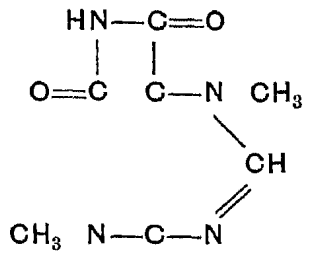
It occurs in the leaves and beans of the Coffee plant (*Coffea arabica*), in leaves of the Tea plant (*Thea sinensis*), in leaves of *Ilex paraguensis* ("Paraguay Tea"), in the fruit of *Paullinia Cupana* and in Kola nuts (*Cola acuminata*)

Expt 158 *Preparation of caffeine from tea*[1] Digest 100 gms of tea with 500 c c of boiling water for a quarter of an hour. Then filter through thin cloth or fine muslin using a hot-water filter in order to keep the liquid hot Wash the residue with a further 250 c c of boiling water Add to the filtrate a solution of basic lead acetate until no more precipitate is formed. This removes proteins and tannins Filter hot and to the boiling filtrate add dilute sulphuric acid until the lead is precipitated as sulphate. Filter from the lead sulphate, and concentrate the solution, with the addition of animal charcoal, to 250–300 c c Filter and extract the filtrate three times with small quantities (50 c c.) of chloroform. Distil off the chloroform on a water-bath, and dissolve the residue in a small quantity of hot water On allowing the solution to evaporate very slowly, long silky needles of caffeine separate, which may have a slightly yellow tint, in which case they should be drained, redissolved in water, and boiled with the addition of animal charcoal The yield should be about 1·5 gm.

Evaporate a little of the caffeine on a water-bath with bromine water A reddish-brown residue is left which becomes purple when treated with ammonia

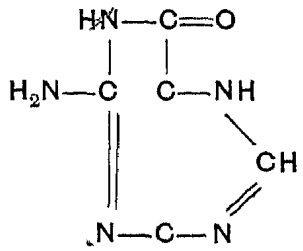
[1] From Conen, *Practical Organic Chemistry*

Theobromine is 3, 7-dimethylxanthine

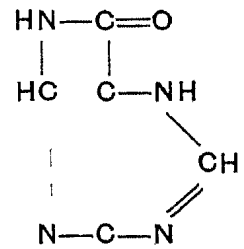


It occurs in the fruit of the Cocoa plant (*Theobroma Cacao*), i leaves of the Tea plant (*Thea sinensis*) and in the Kola nut (*Col acuminata*).

Guanine and **hypoxanthine** can be represented respectively a 2-amino, 6-oxypurine and 6-monoxypurine.



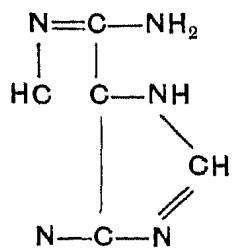
Guanine



Hypoxanthine

They usually occur together and have been found in the germinatin seeds of the Sycamore (*Acer pseudoplatanus*), Pumpkin (*Cucurbi Pepo*), Common Vetch (*Vicia sativa*), Meadow Clover (*Trifoliu pratense*), yellow Lupin (*Lupinus luteus*) and Barley (*Hordeum vulgare* also in the juice of the Beet (*Beta*).

Adenine is 6-aminopurine. It is represented as



It has been found in Beet (*Beta*), Tea leaves (*Thea sinensis*) and leaves of the Dutch Clover (*Trifolium repens*).

Guanine, hypoxanthine and adenine are all obtained by the hydr lysis of plant nucleoproteins.

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